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Relationship between paraoxonase 1 activity and high density lipoprotein concentration during naturally occurring babesiosis in dogs

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ABSTRACT

Paraoxonase 1 (PON1) is a negative acute phase protein bound to high density lipoproteins (HDL) and during the acute phase response (APR) protects HDL from peroxidation. The aim of this study was to assess the relationship between PON1 and HDL in canine babesiosis, a disease characterized by oxidative damages and by an APR. PON1, HDL and C-reactive protein (CRP), were measured in blood collected from 15 controls and 29 dogs with babesiosis sampled at admission, and on days 1 and 7 after treatment. At admission, PON1 and HDL were significantly lower in affected dogs. HDL concentration increased at day 1 while PON1 increased and CRP decreased at day 7. This suggests that the decrease of PON1 at admission is in part due to an increased consumption, the decreased HDL may depend on lipid peroxidation and its rapid increase after treatment may depend on the antioxidant activity of PON1.

1. Introduction

Canine babesiosis is a tick-borne disease caused by intra-erythrocytic protozoa belonging to the genus *Babesia*, with a worldwide distribution (Boozer and Macintire, 2003). Different species have been identified, with *Babesia canis*, *Babesia rossi*, *Babesia gibsoni*, *Babesia vogeli* and the *Babesia microti-like* piroplasm being the most diffuse, depending on geographical distribution of vectors. Canine babesiosis caused by *B. canis* is a very common cause of morbidity and mortality of dogs in the Mediterranean area (Solano-Gallego and Baneth, 2011). The disease can be clinically classified into uncomplicated and complicated forms characterized by acute hemolytic anemia and by a systemic inflammatory response syndrome, respectively. Despite the variability in clinical signs, *B. rossi* is usually associated with severe systemic involvement, while *B. canis* is usually considered less virulent and associated mainly with erythrocyte damages. Dogs with uncomplicated babesiosis are usually presented with pale mucous membranes, fever, anorexia, and splenomegaly (Solano-Gallego and Baneth, 2011). The pathogenesis involves the direct mechanical destruction of parasitized erythrocytes, an immune-mediated hemolysis and the systemic inflammatory response. This has been hypothesized based on the difference between the severity of anemia and the magnitude of parasitemia, that are not proportional to each other (Carson and Philips, 1981; Kawamura et al., 1987; Murase and Maede, 1990). Several studies demonstrated that non-parasitized erythrocytes may also be damaged, most likely due to a multifactorial pathogenesis including immune-mediate processes (Morita et al., 1996), oxidative damages of erythrocytes (Chaudhuri et al., 2008; Murase et al., 1996) and a systemic acute inflammatory response (Matijatko et al., 2007).

In vitro, it has been demonstrated that blood monocytes from *B. gibsoni*-infected dogs release a significantly higher amount of reactive oxygen intermediates as well as lipid peroxidation products of erythrocyte membranes (Otsuka et al., 2002). The oxidative damage of erythrocytes is induced by the replication of parasites (Murase et al., 1996). Moreover, increased erythrophagocytic activity of macrophages has been observed (Murase and Maede, 1990) and this contributes to induce oxidative damages of the erythrocytes (Otsuka et al., 2002). Also the acute phase response (APR) that develops during babesiosis contributes to the release of oxidative compounds and to macrophage activation. The APR leads to a systemic inflammatory response syndrome (SIRS) associated with a multiple organ dysfunction syndrome (MODS) only in complicated forms, most commonly related to *B. rossi* and rarely to *B. canis* (Jacobson, 2006; Solano-Gallego et al., 2008). Nevertheless, an APR is also present in uncomplicated forms, as demonstrated by the increase of acute phase protein, such as C reactive protein (CRP), serum amyloid A (SAA) and, less constantly, haptoglobin in dogs with

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babesiosis. CRP and SAA rapidly decrease after treatment. Therefore, these two acute phase proteins (APP) may be useful in treatment monitoring (Matijatko et al., 2007).

Paraoxonase 1 (PON1) is a calcium-containing enzyme synthesized by liver, belonging to the family of fast negative acute phase protein with antioxidant properties (Feingold et al., 1998). PON1 circulates in blood bound to high density lipoproteins (HDL) and protects low density lipoproteins (LDLs) and HDL from peroxidation (Aviram et al., 1998; Mackness et al., 2004). Moreover, PON1 possesses anti-inflammatory properties, as it reduces the production of pro-inflammatory mediators (Watson et al., 1995). Specifically, PON1 is tightly associated with apolipoprotein A1 in HDL and exerts its protective function through an arylesterase activity that hydrolyzes oxidized lipids. During an acute phase response, HDL molecules lose apolipoprotein A1, esterified cholesterol, and most of the HDL-associated enzymes, including PON1, which is replaced mainly by serum amyloid A and ceruloplasmin (Watson et al., 1995). Paraoxonase activity has been already evaluated in serum of dogs affected by leishmaniosis, as a model of chronic inflammation characterized by oxidative phenomena (Rossi et al., 2014). However, in leishmaniotic dogs, PON1 is low only when severe clinical signs and a systemic inflammatory response are present. In these dogs, PON1 normalizes earlier than other markers after successful treatment. Recently, it has been demonstrated that in dogs with leishmaniosis also the serum concentration of HDL is low at admission and increases after therapy supporting the hypothesis of an interaction between PON1 and HDL during chronic inflammation (Ibba et al., 2013). Recently, a decrease of HDL in dogs with babesiosis followed by an increase after treatment has been reported (Mrljak et al., 2014). However the possible correlation between HDL and oxidants or antioxidants molecules has not yet been investigated in dogs with babesiosis.

Contrarily to leishmaniosis, babesiosis could be an ideal model of acute inflammation associated with a severe oxidative process. Therefore, PON1 and HDL could be early markers that reflect both the inflammation and the oxidative status. Thus, the aim of this study is to assess the relationship between the changes of PON1 and HDL in dogs with canine babesiosis at admission and during treatment in order to assess the antioxidative role of PON1 during a disease characterized by acute inflammation-oxidation and the possible role of both the analytes as a marker for treatment monitoring.

2. Materials and methods

2.1. Animals and study design

This was a prospective study performed on dogs naturally affected by babesiosis and referred to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia. Inclusion criteria were: (1) clinical signs consistent with acute babesiosis (e.g. pale mucous membranes, fever, anorexia, depression, splenomegaly and water hammer pulse); (2) final diagnosis confirmed by demonstration of the parasites within the infected erythrocytes in Romanowsky-stained thin blood smears followed by PCR to correctly identify *Babesia* species; (3) absence of previous or undergoing treatments against babesiosis; (4) no concurrent treatments administered; (5) absence of clinical signs or laboratory changes consistent with metabolic or endocrine diseases; (6) negative serology test for leishmaniosis and ehrlichiosis (i.e. antibody titers equal or lower than 1:40, that is considered the threshold of positivity at the laboratory of veterinary teaching hospital for both the diseases).

Additionally we included, as negative controls, clinically healthy dogs referred to the diagnostic laboratory of the Department of Veterinary Sciences and Public Health (Divet-lab), University of Milan, Italy. Inclusion criteria for control dogs were: (1) negative serology for canine leishmaniosis and ehrlichiosis; (2) absence of clinical or laboratory abnormalities. These dogs were sampled during routine wellness visits under informed owner consent. According to our Institution's regulations, when informed owner consent is obtained, a formal approval from the Ethical Committee is not required if samples are performed for diagnostic or monitoring purposes, as in this case.

At admission (day 0) dogs with babesiosis received a complete physical examination. Peripheral blood was collected from jugular or cephalic vein and immediately transferred in part into a tube containing EDTA and in part into a plain tube (Becton, Dickinson and Co., Rutherford, NJ, USA). Then, one dose (6 mg/kg) of imidocarb dipropionate (Imizol, Schering-Plough, Kenilworth, NJ, USA) was administered to all the dogs with babesiosis subcutaneously. When possible, samplings were repeated during the follow-up and analyzed for PON1 ($n = 28$), HDL ($n = 27$) and CRP ($n = 28$). Specifically, blood samples were collected 24 hours after treatment (day 1), and 1 week after treatment (day 7).

For all the samples collected at admission or during the follow up, immediately after sampling, routine hematology was performed on anticoagulated blood using the ABC Vet hematology analyzer (ABX Diagnostics, Montpellier, France).

In 26 cases, serum obtained by centrifugation (1100 $g \times 8$ min) of blood collected in plain tubes was used to run a panel of biochemical tests (urea, creatinine, protein, albumin, total bilirubin, glucose, cholesterol, triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), creatine kinase (CK), calcium, amylase, lipase, and phosphorus) using an automated analyzer (Olympus AU 600, OlympusDiagnostica GMBH, Hamburg, Germany). In the remaining four cases biochemical tests were not run due to the insufficient amount of serum available.

The health status of treated dogs was monitored over a 6 month period by referring veterinarians.

2.2. Molecular analysis

DNA was extracted from 200 μL of EDTA-treated blood using the DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of a fragment (~560 bp) of the ssrRNA gene was obtained by using the forward primer 5′-GTCTTGTAATTGGAATGATGG-3′ and the reverse primer 5′-CCAAAGACTTTGATTTCTCTC-3′, specific for *Babesia* spp. A plasmid containing an ssrRNA gene fragment was used as positive control in each round of amplification. The same plasmid was used to spike PCR-negative samples to exclude lack of amplification due to the presence of inhibitors in the extracted DNA. The PCR mixture consisted of 10 μL of 5× PCR buffer polymerase (Promega, Madison, WI, USA), 1.5 mM MgCl2, 200 μM of each dNTP, 10 pmol of each primer and 1.0 U of GOTaq DNA (Promega) in a volume of 45 μL, to which 5 μL of extracted DNA were added. Amplification was performed on a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). An initial denaturation step at 94 °C for 2 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 60 s. A final extension was done at 72 °C for 7 min, followed by a hold step at 4 °C. Amplified DNA was subjected to electrophoresis in 1% or 1.5% agarose gel (100 V, 60 min), pre-stained with ethidium-bromide and viewed under UV light (Beck et al., 2009).

2.3. Serology

The presence of anti-*Ehrlichia canis* and anti-*Leishmania infantum* antibodies in canine serum was investigated by indirect fluorescent antibody test (IFAT) using commercial IFAT kits, following the manufacturer's instructions (*Ehrlichia canis* IgG IFA kit, Fuller