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Iron metabolism and oxidative profile of dogs naturally infected by *Ehrlichia canis*: Acute and subclinical disease

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ABSTRACT

The aim of this study was to evaluate the oxidant profile and iron metabolism in serum of dogs infected by *Ehrlichia canis*. Banked sera samples of dogs were divided into two groups: negative control ($n = 17$) and infected by *E. canis* on acute ($n = 24$), and subclinical ($n = 18$) phases of the disease. The eritrogram, leucogram, and platelet counts were evaluate as well as iron, ferritin, and transferrin levels, latent iron binding capacity (LIBC), and transferrin saturation index (TSI) concentration. In addition, the advanced oxidation protein products (AOPP) and ferric reducing ability of plasma (FRAP) in sera were also analyzed. Blood samples were examined for the presence of *E. canis* by PCR techniques. History and clinical signals were recorded for each dog. During the acute phase of the disease, infected animals showed thrombocytopenia and anemia when compared to healthy animals ($P < 0.05$) as a consequence of lower iron levels. Ferritin and transferrin levels were higher in both phases (acute and subclinical) of the disease. The AOPP and FRAP levels increased in infected animals on the acute phase; however, the opposite occurred in the subclinical phase. We concluded that dogs naturally infected by *E. canis* showed changes in the iron metabolism and developed an oxidant status in consequence of disease pathophysiology.

1. Introduction

Canine monocytotropic ehrlichiosis (CME) is an important disease with a worldwide distribution. Although CME has been considered specie-specific, studies have shown that this obligate intracellular gram-negative bacterium *Ehrlichia canis* transmitted by ticks *Rhipicephalus sanguineus* [1,2], may also infect other species besides dogs, including man [3].

CME is considered an endemic disease in Southeastern Brazil, showing acute, subclinical, and chronic phases, which are classified according to clinical signs and clinic-pathological abnormalities [3,4]. The acute phase is easily recognizable due to its clinical manifestation that includes: fever, weight loss, anorexia, bleeding disorders, lymphadenomegaly, anemia thrombocytopenia, and leucopenia [5–8]. On the other hand, the subclinical phase shows significant variable length, extending from months to years [9].

Traditional diagnostic techniques (hematology, cytology, serology, and isolation) are considered valuable tools for CME diagnosis. However, the immune response and the oxidant profile of dogs with CME infection also seem to play a central role in disease pathogenesis, especially when it is observed different clinical

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signs and/or laboratory and pathological findings [7,10].

Previous studies demonstrated that early endosomes containing different species of *Ehrlichia* (morulae) upregulate and accumulate the mammalian transferrin receptor [11], suggesting that *Ehrlichia* spp. has developed strategies for its own iron (Fe) acquisition. Besides other functions, Fe is directly involved with electron transport (cytochromes) and oxygen activation (oxidase and oxygenases), and transportation (hemoglobin and myoglobin) [12], and it is, consequently, important in the mechanism of anemia development. By presenting this information, the aim of this study was to evaluate the hematological parameters, iron metabolism, and oxidative profile in serum samples from dogs naturally infected by *E. canis*.

2. Materials and methods

2.1. Animals

Sera samples were collected from dogs naturally infected by *E. canis*; twenty four (24) on the acute phase of the disease (with clear clinical signs) and eighteen (18) with subclinical disease (asymptomatic). As a control group, sera samples from seventeen (17) healthy dogs were used.

Animal history, clinical signs, complete blood count (CBC), and biochemical profile were analyzed and recorded for each dog. Blood samples from all dogs were tested for the presence of *E. canis*, *Babesia canis*, *Babesia gibsoni,* and *Anaplasma plattys* by nested PCR [13–15].

2.2. Hemogram

Blood was collected and stored in tubes with EDTA for CBC. Blood samples were evaluated using an automated blood cell counter (ABC Vet. Horiba ABX – São Paulo, Brazil) to determine red blood cell (RBC) count, hemoglobin concentration (Hb), total leukocytes and platelets counts. Hematocrit was assessed by using the standard microhematocrit method (Centimicro mod. 1-15-Sigma, Germany). Blood smears were also prepared and stained for microscopic examination.

2.3. Iron metabolism

Iron (Fe) metabolism was assessed through the evaluation of the following variables: serum iron and latent iron-binding capacity (LIBC), through commercial kits (Labtest, Minas Gerais, Brazil) on a semi-automatic analyzer Bio-2000 (Bio Plus Ltda, São Paulo, Brazil). All glassware used was previously soaked into 10% hydrochloric acid for 3 h and rinsed with deionized water (Milli-Q system from Millipore Corporation). Transferrin and ferritin concentrations were assessed using an automated immunoturbidimetry (Labtest, Minas Gerais, Brazil). Additionally, it was estimated the transferrin saturation index (TSI).

2.4. AOPP and FRAP levels

Protein oxidation status was evaluated through the measurement of AOPP concentrations as described by Hanasand et al. [16]. Levels of ferric reducing antioxidant power (FRAP) was measured according to the technique described by Benzie and Strain [17] in sera samples. AOPP and FRAP results were expressed as μmol L^{-1} according to the modified Griess method using the Cobas Mira automated analyzer.

2.5. Statistical analysis

Firstly, the data were subjected to normality test, where we verified a normal distribution. Then, the data were subjected to Tukey test. Values with probability (p) less than 5% were considered statistically different. Data were presented as mean values ± standard deviation.

3. Results

3.1. Clinical signs and hematological parameters

As expected, control group and subclinically *E. canis* infected animals did not show clinical signs of the disease. However, dogs with *E. canis* acute infection showed several clinical signs, such as: apathy, appetite loss and intermittent fever. It is important to emphasize that more than 40% of the dogs with acute infection were also parasitized by ticks. Serology was negative for *B. canis*, *B. gibsoni* and *A. plattys*.

Peripheral blood smears showed hematological changes in animals infected by *E. canis* (Table 1). According to our results, it was possible to observe that animals with the acute phase the CME had a significant decrease ($P < 0.05$) in erythrocyte, hematocrit, hemoglobin and platelet counts when compared to healthy animals. For erythrocyte, hematocrit, hemoglobin and platelet parameters, no significant differences were observed on *E. canis* infected animals with the subclinical disease compared to healthy animals.

Leukocyte, lymphocyte, neutrophil, eosinophil and monocyte counts did not differ during the acute or subclinical phases of the disease when compared to healthy animals. In addition, the bands number, during the acute phase of disease, were higher in infected animals on the acute phase when compared to infected animals on the subclinical phase of CME and to healthy animals.

3.2. Iron metabolism

Iron, ferritin, transferrin, LIBC, and TSI levels are shown in Table 2. *E. canis* infected animals showed a significant increase of ferritin, transferrin, and TSI levels during the acute phase of CME, when compared with healthy animals. Additionally, Fe level significantly decreased during the acute phase of CME compared to healthy animals. Also, ferritin and transferrin levels increased on animals on subclinical disease in comparison to the control group ($P < 0.05$). There were no differences on Fe and TSI levels on infected animals with the subclinical phase of CME. Furthermore, LIBC serum levels did not differ on *E. canis* infected animals.

Ferritin, transferrin and TSI levels were lower in infected animals during subclinical phase, when compared with animals on the acute phase. However, Fe levels were higher in infected animals on subclinical phase than in infected animals on the acute phase of disease.

3.3. AOPP and FRAP levels

AOPP and FRAP results are shown in Table 2. Animals on acute CME presented increased levels of AOPP and FRAP on sera when compared to healthy animals. Similarly, FRAP levels increased in dogs on the subclinical phase when compared to the control group ($P < 0.05$). There were no differences on AOPP levels in infected animals on the subclinical phase of CME. AOPP and FRAP levels were significantly lower in infected animals on the subclinical disease, when compared to dogs on the acute phase of disease.

Table 1
Eritrogram, leucogram and platelet counts in dogs naturally infected by *Ehrlichia canis*: acute and subclinical disease compared to healthy animals.

Variables	Healthy dogs control	Infected dogs acute phase	Infected dogs chronic phase
Total erythrocytes ($\times 10^6/\mu L$)	5.85 ± 1.22[a]	4.47 ± 1.17[b]	5.64 ± 1.50[ab]
Hemoglobin (g/dL)	13.4 ± 2.4[a]	9.72 ± 2.8[b]	13.3 ± 3.5[ab]
Hematocrit (%)	40.1 ± 7.4[a]	28.4 ± 7.4[b]	38.7 ± 10.1[ab]
Total leukocytes ($\times 10^3/\mu L$)	7.76 ± 2.96[a]	6.80 ± 3.34[a]	8.27 ± 5.5[a]
Lymphocytes ($\times 10^3/\mu L$)	2.25 ± 1.21[a]	2.24 ± 1.42[a]	2.03 ± 0.97[a]
Neutrophils ($\times 10^3/\mu L$)	4.38 ± 1.88[a]	3.69 ± 2.31[a]	5.41 ± 2.96[a]
Bands (/μL)	45.8 ± 72.2[a]	199.5 ± 89.61[b]	60.3 ± 57.2[a]
Eosinophils (/μL)	615.4 ± 534.2[a]	214.4 ± 229.1[a]	377.1 ± 298.2[a]
Monocytes (/μL)	465.6 ± 420.1[a]	455.6 ± 256.4[a]	391.1 ± 420.2[a]
Platelets ($\times 10^3/\mu L$)	294.4 ± 111.4[a]	90.2 ± 64.2[b]	228.3 ± 140.0[a]

Same letter in the same line means that there are no statistical differences between groups. Values with $P < 0.05$ were considered statistically different. Data were presented as mean values ± standard deviation.

Table 2
Iron, ferritin and transferrin levels; latent iron binding capacity (LIBC), transferrin saturation index (TSI) concentrations; advanced oxidation protein products levels (AOPP), and ferric reducing antioxidant power (FRAP) of dogs naturally infected by *Ehrlichia canis* (acute and chronic phases) compared to healthy dogs (control).

Variable	Healthy dogs control	Infected dogs acute phase	Infected dogs subclinical phase
Iron ($\mu g\ dL^{-1}$)	106.31 ± 7.8[a]	78.6 ± 5.9[b]	99.5 ± 6.8[a]
Ferritin ($ng\ mL^{-1}$)	1.25 ± 0.26[a]	8.74 ± 1.58[b]	3.34 ± 1.41[c]
Transferrin ($mg\ dL^{-1}$)	213.6 ± 11.2[a]	412.2 ± 31.4[b]	286.4 ± 19.1[c]
LIBC ($\mu g\ dL^{-1}$)	302.1 ± 21.6[a]	289.7 ± 37.1[a]	321.4 ± 48.1[a]
TSI (%)	37.8 ± 4.1[a]	49.6 ± 7.2[b]	41.4 ± 3.1[a]
AOPP ($\mu mol\ L^{-1}$)	39.0 ± 3.8[a]	56.7 ± 6.1[b]	43.7 ± 4.6[a]
FRAP ($\mu mol\ L^{-1}$)	189.1 ± 48.5[a]	535.1 ± 96.8[b]	379.4 ± 52.4[c]

Same letter in the same line means that there are no statistical differences between groups. Values with $P < 0.05$ were considered statistically different. Data were presented as mean values ± standard deviation.

4. Discussion

This study focused on profiling the oxidative mechanisms and the iron metabolism in sera samples from dogs naturally infected by *E. canis*. Following the normal pattern of CME, infected dogs showed clinical signs such as inappetence, hyperthermia and apathy, as previously described by Munhoz et al. [18]. In order to validate our study data, *E. canis* infection in dogs was confirmed by PCR.

Dogs presenting clinical signs of the infection showed more pronounced laboratorial abnormalities than dogs under subclinical stage. In agreement with these results, some authors consider that alterations in globulin levels can be related to the length of infection [19]. We observed some hematological changes in infected animals on the acute phase, especially anemia and thrombocytopenia. Anemia is a frequent finding in ehrlichiosis [20], similarly to other canine blood diseases such as leishmaniasis, hepatozoonosis and babesiosis [21–23]. In these situations, usually Fe concentration is also reduced. We believe that infected animals in the acute phase of CME, and with number of *E. canis* in infected dogs was high, these developed anemia as result of Fe metabolism. Doyle et al. [24] observed an Fbp 38 kDa protein in *E. canis*, which is involved in the acquisition and transportation of iron. Our results suggest that *E. canis* uses Fe available in the serum in order to maintain its survival, reducing Fe levels in infected animals during the acute phase. This is just a hypothesis, however the mechanism of decrease serum iron levels are not well known in ehrlichiosis, but it is related to high bacteremia, iron therapy in acute infection shouldn't be recommended. *Ehrlichia* upregulates the transferrin receptor and recruits it to the intracytoplasmic vacuole (morula) [24]. It corroborates our findings, since the importance of Fe for intracellular survival and proliferation of *Ehrlichia* have already been demonstrated [11]. Low circulating Fe levels induces the expression of iron binding proteins, such as the extracellular transferrin for Fe transport into tissues, whereas the intracellular ferritin sequestrates Fe. The Fe deficiency may exacerbate the anemia in the myelosuppressive phase of the disease, interfering in the hematopoietic process [20].

Low Fe levels in sera of animals infected by *E. canis* during the acute phase of the disease, associated with high transferrin and normal LIBC levels indicate a higher production of hepatic transferrin, presumably attempting to maximize the use of lower extracellular iron. During subclinical stages, infected animals develop an anemia, similarly to the anemia observed on chronic disease. In these cases, there are normal Fe levels in sera, but the majority of this Fe is associated with ferritin and transferrin, keeping the LIBC and TSI presumably normal, and allowing the resistance mechanism against *E. canis*, especially through upregulating the levels of hepcidin, a hormone produced in liver that regulates Fe homeostasis [25]. Hepcidin is released during the inflammatory process mediated by IL-6, an important cytokine in the immune response against *E. canis* [26]. IL-6 promotes the release of hepcidin in turns, leading to the decrease of seric Fe [25], which causes changes in the immune response, since lymphoid cells are dependent on Fe for cell division, electron transport, oxidation–reduction reactions, and phagocytic activity [25,27].

Regarding the mechanism of thrombocytopenia, it may involve immune destruction (mainly during the acute phase) by increasing platelet consumption, decreasing platelet half-life, splenic sequestration or, it may be secondary to increased concentrations of circulating platelet migration-inhibition factor [28,29]. The thrombocytopenia is the hallmark of acute stage and it is usually characterizing by the presence of megaplatelets in the peripheral blood [28]. Our results showed exactly this pattern, since thrombocytopenia was found in dogs under acute infection, but not on those with the chronic form of the disease or healthy. There is also the hypothesis that antiplatelet antibodies also act on bone marrow megakarocytes with deleterious effects on thrombopoiesis [30], collaborating with the process of platelets depletion in dogs acutely affected by *E. canis*.

It is possible to correlate our hematological results to oxidative damage to red blood cells, a process related to the generation of free

radicals [31]. Our data show increased levels of AOPP and FRAP, both variables in sera of infected animals with *E. canis* during the acute phase. These are antagonistic biomarkers, since increased levels of AOPP indicates the enhancement of protein oxidation [32], while FRAP measures the total antioxidant capacity (TAC) [17]. Enhanced oxidative stress reduces erythrocyte deformability [33,34], contributing to hemolysis, and to the development of anemia [35]. It was already reported that AOPP has direct relation with anemia in malaria [36]. Furthermore, *E. canis* plays an oxidative stress through lipoperoxidation [37] contributing to high levels of AOPP. Thus, due to the increase of AOPP levels observed during the acute phase of CME in our study, it is possible to assume that there was a biologic response provided by FRAP increase, as an attempt to provide a balance between oxidant and anti-oxidant mechanisms. Additionally, these results indicate a direct effect on NO levels, since Fe plays an inhibitory role on the expression of inducible NO synthase (iNOS). There were no differences on AOPP levels between control group and animals on subclinical phase of CME, indicating reduction on hematological damage. However, FRAP levels still increased in animals under acute and subclinical phase, when compared to the control group. Therefore, even without protein oxidation in subclinical animals, the total antioxidant capacity still increased when compared to the control group, indicating a long-term antioxidant activity. Also, reduction AOPP and FRAP levels, on subclinical phase of CME show a restoration of serum iron.

Positive results from PCR for *E. canis* validated our model and reinforced our results. The main changes observed were found during the acute phase of the disease. During this phase, it was observed that *E. canis* infection lead to anemia and thrombocytopenia, and we correlated this anemia mainly to iron metabolism. Added to these hematological alterations, we observed increased FRAP and AOPP levels. Therefore, our results indicated hematological and oxidative imbalance, especially during the acute phase of ehrlichiosis.

5. Commission of ethics and animal welfare

The present study was approved by the Ethics Committee for Use of Animals (CEUA) of São Paulo State University (UNESP), Jaboticabal Campus, under protocol number 010290/14.

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