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Serum antioxidant capacity and oxidative damage in clinical and subclinical canine ehrlichiosis

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ABSTRACT

The objective of this study was to evaluate and compare the antioxidant response and the products of oxidative damage analysed by various assays in clinical and subclinical canine monocytic ehrlichiosis (CME). For this purpose, four assays to measure the total serum antioxidant capacity (TAC), such as the cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC) using acidic medium ($TEAC_A$), and the TEAC using the horseradish peroxidase ($TEAC_H$) were used. In addition, the serum thiol concentrations were analysed. Reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), and ferrous oxidation-xylenol orange (FOX) were measured to determine the concentrations of free radical and the products of oxidative damage as result of the disease. All antioxidant markers were significantly lower in the dogs on clinical ehrlichiosis when compared with healthy dogs; however only the CUPRAC, FRAP and thiol were significantly lower in subclinical CME compared with healthy dogs. TBARS and FOX showed no significant differences between dogs with CME and healthy dogs; however, a significant increased ROS concentration was observed in dogs with clinical and subclinical CME when compared with healthy dogs. Results showed that in CME there is a state of oxidative stress with significant changes in markers of antioxidant defence and in concentrations of free radicals. However, the detection of these changes would depend of the assay used.

1. Introduction

Canine monocytic ehrlichiosis (CME) is a tick-borne disease caused by the intracellular bacteria *Ehrlichia canis*, widely distributed around the world. The course of the disease can be divided into three phases: acute, subclinical and chronic. The acute phase occurs after an incubation period of 8–20 days and persists for 2–4 weeks, during which there is a multiplication of the bacteria in mononuclear cells and a spread within the host. The main clinical signs in the acute phase of the disease are fever, weight loss, anorexia, lymphadenomegaly, anaemia with or without bleeding disorders such as petechial and/or dermal ecchymosis (Nair et al., 2016; Shaw et al., 2001). Following the acute phase, the infection may persist after spontaneous clinical recovery or ineffective treatment, and such animals may enter the subclinical stage of CME where no clinical signs are evident (Harrus et al., 1997; Skotarczak, 2003; Waner et al., 1997). In the chronic phase, dogs can develop pancytopenia associated with hypoplastic bone marrow failure leading to death (Saito and Walker, 2016).

Oxidative stress is implicated in the pathogenesis of CME infection (Bottari et al., 2016; Da Silva et al., 2013; Rudoler et al., 2015). Previous studies demonstrated increased lipid and protein oxidation in dogs experimentally infected by *E. canis* through high levels of thiobarbituric acid reactive substances (TBARS) and advanced oxidation protein products (AOPP), respectively (Bottari et al., 2016; Da Silva et al., 2013). Regarding antioxidant status, studies showed that dogs with ehrlichiosis presented decreased total serum antioxidant capacity (TAC) concentrations when measured by the trolox equivalent antioxidant capacity ($TEAC_A$) (Rudoler et al., 2015). However, another report described dogs with clinical CME who presented increased TAC levels when measured by the ferric reducing ability of plasma (FRAP)

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(Bottari et al., 2016).

The main aim of this study was to evaluate and compare serum antioxidant capacity and products of oxidative damage in clinical and subclinical ehrlichiosis and in healthy dogs. A special emphasis will be given to the study of possible differences in serum TAC when measured using four different assays: cupric reducing antioxidant capacity (CUPRAC), FRAP, TEAC using an acidic medium ($TEAC_A$), and TEAC using the horseradish peroxidase ($TEAC_H$). In addition, total serum thiol was evaluated for the first time in this disease as a biomarker of oxidative stress. Regarding oxidant content, the reactive oxygen species (ROS) production, TBARS and ferrous oxidation-xylenol orange (FOX) levels were analysed and compared. Furthermore, a second objective of this work was to study the possible influence of inflammation in oxidative stress in CME by the analysis of the correlations between the serum biomarkers of oxidative stress and markers of the inflammation such as C-reactive protein (CRP) and ferritin.

2. Material and methods

2.1. Animals and sampling procedures

Serum samples were collected from thirty dogs admitted to the Veterinary Hospital of Uludag University, Bursa, Turkey. The dogs were divided into three groups: clinical ehrlichiosis, subclinical ehrlichiosis and a control group. Data of the breeds, age, physical examination, analytical evaluation and serology of the dogs in each study group appears in Table 1. As inclusion criteria, all dogs should have had no evidence of concurrent vector-borne or non-infectious systemic diseases, being negative for *Dirofilaria immitis* antigen, antibodies against *Anaplasma phagocytophilum* and *Anaplasma platys*, negative for antibodies against Leishmania with the Anigen Rapid CaniV-4 (Bionote, Korea), and not having received any previous treatment at the time of sampling. The dogs of the control group were considered healthy after physical examination, and haematological and biochemistry evaluations during routine check-ups or vaccinations. In addition, they were seronegative to *E. canis* (Anigen Rapid CaniV-4 test kits, Bionote).

Blood samples were obtained from the cephalic vein. Immediately after collection, the blood was placed in special tubes with a coagulation activator and a gel separator (Tapval, Aquisel, Spain). The samples were centrifuged at 3500 × *g* for 5 min and the sera obtained were stored frozen (− 80 °C) until analysis. The study was approved by the Uludag University ethics committee (24.09.2013/2013–14/07).

2.2. Antioxidant capacity

The CUPRAC assay was performed as described by Campos et al. (2009) and previously validated for use in dog serum (Rubio et al., 2016a). The FRAP assay was based on that described by Benzie and Strain (1996) and previously used in dogs (Heaton et al., 2002; Hetyey et al., 2007). The $TEAC_A$ and $TEAC_H$ assays were performed using the assays described by Erel (2004) and Arnao et al. (1996), respectively and previously validated for use in serum from dogs (Rubio et al., 2016b). Total serum thiol concentration was measured according to the method described by Jocelyn (1987) and Costa et al. (2006). All analyses were performed in the Olympus AU600 (Olympus AU600 Automatic Chemistry Analyser, Olympus Europe GmbH, Germany), and all assays showed intra- and inter-assay imprecision lower than 6% and 13%, respectively.

2.3. Products of oxidative damage

ROS levels were quantified by luminol-mediated chemiluminescence assay (Vong et al., 2014). The resulting chemiluminescence was measured using a microplate reader (Victor 2 1420 Multilabel Counter; PerkinElmer, Finland) and expressed in counts per second (cps). TBARS levels were measured as described by Buege and Aust (1978). Final absorbance of the samples was measured by use of a microplate reader (Powerwave XS, Biotek instruments, Carson City, NV). FOX assay was based on the colorimetric method described by Arab and Steghens (2004) and was performed using the Olympus AU600 Automatic Chemistry Analyser. All assays showed inter- and intra-assay imprecision lower than 15%.

2.4. Inflammatory markers

CRP concentrations were measured using an immunoturbidimetric assay (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Hamburg, Germany) with a method previously validated for use in dogs (Martínez-Subiela and Cerón, 2005). Ferritin concentrations were measured using a commercial immunoturbidimetric assay (Tinaquant Ferritin, Roche) previously validated for use in dog serum (Martinez-Subiela et al., 2014). All analyses were performed in serum using the Olympus AU600.

2.5. Statistical analysis

The Graphpad Prism software was used for all data analysis. Because of the small sample size, the changes in variables between

Table 1
Characteristics of the study groups.

	Clinical Ehrlichiosis (n = 10)	Subclinical Ehrlichiosis (n = 10)	Healthy (n = 10)
Breed	-1 Anatolian shepherd dog -1 Belgian shepherd -4 Golden retrievers -1 Labrador retriever -1 Pointer -2 mixed breed dogs	-1 Anatolian shepherd dog -1 Golden retrievers -1 German shepherd -2 Pointer -5 mixed breed dogs	-3 Beagles -3 Golden -4 mixed breed
Median age (range)	5 years (1 − 11)	5 years (1–11)	6 years (1–11)
Physical examination findings	At least two of the following: -Weight loss -Anorexia -Ecchymosis	None	None
Haematological changes	At least two of the following: -Anaemia -Thrombocytopenia -Leukopenia	None	None
Serology	High seropositive to *E. canis*[*]	Seropositive to *E. canis*[*]	Negative to *E. canis*

[*] Anigen Rapid CaniV-4 test kits, Bionote.