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Lipid peroxidation in dogs naturally infected with *Babesia canis canis*

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ABSTRACT

The possible role of oxidative stress in the pathogenesis of infections caused by parasites has been an active area of research in recent years. The aim of this study was to assess changes in activity concentrations of malondialdehyde (MDA), a commonly used biomarker for assessing lipid peroxidation, in dogs infected with *Babesia canis canis*, after therapy with imidocarb dipropionate, and to compare them with MDA concentrations prior to treatment and to healthy controls. The study was conducted on a sample of 30 dogs suffering from babesiosis and 25 healthy dogs (control group). Blood was taken before treatment, on the first day (all dogs) and on the second, third, and seventh days of the research (affected dogs and ten healthy dogs). Application of imidocarb diproprionate in ten healthy dogs did not affect the values of MDA, so in our study all healthy dogs were used as controls. On the first day of the research the MDA concentrations were significantly increased in dogs with babesiosis ($P<0.001$) in comparison with the control group. On the seventh day MDA decreased, but was still significantly higher compared to the control group ($P<0.001$). Concentrations of MDA on the first day were significantly increased in dogs with uncomplicated ($P<0.001$) and complicated babesiosis ($P<0.001$) compared to controls. On the seventh day the concentration of MDA was significantly higher in the group of dogs with complicated babesiosis ($P = 0.003$) than in the uncomplicated group. Furthermore, dogs in the multiple organ dysfunction syndrome group had significantly increased MDA concentrations compared to dogs in the uncomplicated ($P = 0.006$) and single complication groups ($P = 0.025$). There was a significant positive correlation between MDA concentration and the outcome of the disease (Tau-b 0.309; $P = 0.005$). The present study concludes that, on the seventh day, increased lipid peroxidation was still present in the affected group of dogs. The results indicate that oxidative stress could have a possible causative role in the clinical severity of the disease.

Key words: oxidative stress, malondialdehyde, uncomplicated babesiosis, complicated babesiosis

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Introduction

Canine babesiosis is a protozoal, tick born disease with worldwide distribution and global significance (SOLANO-GALLEGO and BANETH, 2011). In Europe, the disease is caused by the intra-erytrocytic protozoal parasites *Babesia canis*, *Babesia gibsoni* and *Babesia microti-like* species. There are three genetically distinct subspecies of *Babesia canis*: *Babesia canis canis, Babesia canis vogeli* and *Babesia canis rossi* (UILENBERG et al., 1989; TABOADA and MERCHANT, 1991; CAMACHO et al., 2001). Canine babesiosis caused by *Babesia canis canis* (*B. canis canis*) is a common cause of morbidity among dogs in Croatia, particularly around the city of Zagreb (CACCIO et al., 2002; MATIJATKO et al., 2007; BECK et al., 2009; BRKLJACIC et al., 2010).

Babesiosis in dogs is, by its clinical manifestation, generally divided into uncomplicated and complicated forms (JACOBSON and CLARK, 1994). The clinical presentation is diverse and ranges from transient anorexia to a complex syndrome in which multiple organ systems are affected. The pathophysiology of canine babesiosis has been extensively studied, but many questions remain unanswered (MATIJATKO et al., 2012). Recent research has indicated that both uncomplicated and complicated forms are associated with host inflammatory responses (MATIJATKO et al., 2007; SCHETTERS et al., 2009; BRKLJAČIĆ et al., 2014). The main mediators of this responses are cytokines, nitric oxide, oxygen free radicals, eicosanoids and platelet activating factor (PURVIS and KIRBY, 1994).

The possible role of oxidative stress in the pathogenesis of infections caused by parasites has been an active area of research in recent years (OTSUKA et al., 2001; BILDIK et al., 2004; ASRI REZAEI and DALIR-NAGHADEH, 2006; CRNOGAJ et al., 2010). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are powerful oxidants and nitrating species that can inactivate enzymes and initiate lipid peroxidation and nitration, which in turn leads to free-radical chain reactions that further damage proteins, membranes and nucleic acids (MULLER et al., 2003). Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues (MAGNI et al., 1994). Malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation, is a reliable and commonly used biomarker for assessing lipid peroxidation (MOORE and ROBERTS, 1998). Determination of MDA allows detection of the degree of lipid peroxidation and the concentration of free oxygen radicals indirectly (DEGER et al., 2009). Serum MDA concentration has been found to be elevated significantly in canine babesiosis (CRNOGAJ et al., 2010).

Despite all the above, there is a limited number of studies and experimental data available on free radicals, oxidative stress markers and antioxidants in dogs with babesiosis. The aim of this study was to assess changes in concentrations of MDA in dogs infected with *B. canis canis* after antiparasitic therapy, and to compare them with MDA

concentrations prior to treatment and to healthy controls. To the best of our knowledge the dynamics of serum MDA concentration after antibabesial treatment has not been reported previously in dogs infected with *B. canis canis.*

Materials and methods

Animals. The study was performed on 55 dogs that were divided into three groups. Thirty dogs naturally infected by *B. canis canis* were included in Group 1. Dogs in this group were aged between one and 13 years, of various breeds and sex. All the dogs infected by *B. canis canis* were presented at the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia, with clinical signs of acute babesiosis. The diagnosis was confirmed by microscopic examination of May-Grünwald Giemsa stained peripheral blood smears, with the finding of large pyriform parasites within the infected erythrocytes. Polymerase chain reaction analysis confirmed the presence of *B. canis canis* subspecies in all dogs. PCR was performed as previously described (BECK et al., 2009). A single dose (6 mg/kg of body weight, subcutaneously) of imidocarb dipropionate (Imizol® 12 %, Schering-Plough) was administered to all the dogs as soon as the diagnosis of babesiosis was confirmed. Twenty-four hours after therapy parasites were not detectable in peripheral blood smears.

On the basis of clinical manifestations and laboratory data the affected dogs were divided into two groups: complicated and uncomplicated babesiosis (JACOBSON and CLARK, 1994). Babesiosis was classified as complicated if one of the following criteria were fulfilled: renal dysfunction (serum creatinine concentration activity of more than 180 μmol/L), hepatic dysfunction (both alanine aminotransferase (ALT) activity greater than 176 U/L and alkaline phosphatase (AP) activity greater than 360 U/L), respiratory system dysfunction (radiographic evidence of pulmonary oedema or dyspnoea), central nervous system dysfunction (a modified Glasgow coma scale less than nine) (WELZL et al., 2001), muscular involvement (creatine phosphokinase (CPK) activity more than 600 U/L) and secondary infection. We included bilirubin serum concentration activity greater than 100 μmol/L as an additional criterion for hepatic dysfunction (WEISER, 1992, MATIJATKO et al., 2009).

Furthermore, on the basis of the number of complications, the complicated group was divided into dogs that developed one complication and dogs that developed MODS.

Group 2 consisted of 25 clinically healthy dogs of various breeds and sex, with a similar age distribution to the infected dogs (one-12 years). The dogs were deemed healthy on the basis of clinical and laboratory data.

Group 3 consisted of ten dogs from group 2 (five males and five females, similar ages). Imidocarb diproprionate (6 mg/kg of body weight, subcutaneously) was administered to all of them as a preventive measure against babesiosis at the request of their owners.

Statistical analysis showed that application of imidocarb diproprionate in healthy dogs did not affect the values of the investigated marker MDA (P = 0.767) throughout all studied days. Therefore in our study we compared all the healthy dogs (group 2) with the infected ones (group 1).

All dogs included in this study were clinically and neurologically examined on the day of admission before therapy with imidocarb dipropionate (first day). Dogs from groups 1 and 3 were also clinically and neurologically examined on the second, third, and seventh days of the research.

Twenty-five dogs (group 2) fulfilled the selection criteria for healthy, control animals and were included in the study.

Thirty cases (group 1) fulfilled the selection criteria for acute canine babesiosis and were included in the study. Polymerase chain reaction analysis confirmed the presence of *B. canis canis* subspecies in all dogs. Uncomplicated babesiosis was established in 22 (73 %) of the 30 dogs, and complicated babesiosis in the remaining eight (27 %). Among the dogs with complicated babesiosis, five (63 %) had single organ dysfunction and three (37 %) dogs had MODS. The numbers of organs involved in dogs with MODS were: four organs (1/3), three organs (1/3) and two organs (1/3).

The mortality rate in this study was 10 %, and all the dogs that died had a complicated form of babesiosis with MODS.

Blood analyses. The blood samples were collected from the cephalic vein on the day of admission, before therapy with imidocarb dipropionate - on the first day (groups 1, 2 and 3) and on the second, third, and seventh days of the research (groups 1 and 3).

The samples were placed in tubes with Ethylenediaminetetraacetic acid (EDTA) for haematological analysis and tubes with no anticoagulant, which were centrifuged at 1500 x g at 4 °C for 10 min. The obtained serum was partly used for analyzing the biochemistry profile and partly stored at -80 °C until it was used for analyzing MDA. Complete blood count was analyzed using an automatic haematology analyzer (Horiba ABX; Diagnostics, Montpellier, France). The biochemistry panel was performed according to standard methods, using an automated biochemistry analyzer (Olympus AU 600, Olympus Diagnostica GMBH). The biochemistry panel included the following parameters: serum creatinine, total protein (TP), albumin, ALT, AP, γ-glutamyl transferase (GGT), blood glucose, total bilirubin and CPK.

Lipid peroxidation was assayed by measuring the serum thiobarbituric acid reactive substances (TBARS) according to the method of TROTTA et al. (1982). MDA is formed as a secondary product when thiobarbituric acid (TBA) and polyunsaturated fatty acid are heated in an acidic medium. Its absorbance was measured at 523 nm on a Thermospectronic Helios delta spectrophotometer (Unicam, Cambridge, UK).

Statistical analysis. Given the nonparametric distribution of quantitative values descriptive statistics were performed and the results presented as the median and interquartile range. The results were analyzed by the Mann- Whitney U-test, and a P value <0.05 was considered to be significant. The relationship between MDA and outcome was assessed by using the Tau-b correlation. The computer software IBM SPSS Statistics version 19.0.0.1. was used for analysis (www.spss.com).

Results

On the first day of the study MDA concentrations were significantly increased in dogs with babesiosis (P<0.001) in comparison to the control group (Fig. 1). Simultaneously, MDA concentrations were significantly increased in dogs with uncomplicated (P<0.001) and complicated babesiosis (P<0.001) compared to the controls, but there were no significant difference in MDA between the uncomplicated and complicated groups (P = 0.122) (Fig. 1). Furthermore, dogs in the MODS group had significantly increased MDA concentrations compared to dogs in the uncomplicated (P = 0.006) and single complication groups (P = 0.025). There was no significant difference in the investigated parameters between dogs in the uncomplicated and single complication groups (Fig. 2).

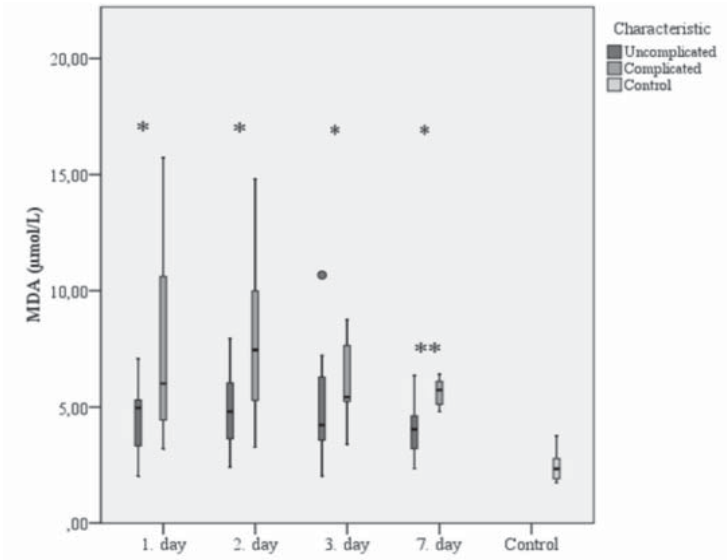

Fig. 1. Malondialdehyde concentration in studied group of dogs infected with *B. canis canis* before (1st day) and after antibabesial treatment (2nd, 3rd and 7th day) of research and control group. * The uncomplicated as well as complicated babesiosis group are significantly different than the control group (P<0.001). ** The complicated babesiosis group is significantly different than the uncomplicated babesiosis group (P 0.003). Plots show the median (dash within box), 25th and 75th percentiles (box), and range (whiskers).

The dynamics of MDA concentrations after treatment: on the seventh day the concentrations of MDA in the affected group were lower than on the first day, but not significantly, and were still statistically higher compared to the control group (P<0.001) (Fig. 1). The MDA concentrations in dogs from the uncomplicated as well as the complicated group showed the same trend, except that the concentrations of MDA in the complicated group were highest on the second day of the study. On the seventh day the concentrations of MDA were significantly higher in the single complication group of dogs (P = 0.003) than in the uncomplicated group (Fig. 1, 2). The dynamics of MDA concentration in the MODS group could not be assessed because of the insufficient number of dogs after the first day of the study (two dogs died on the first day of the study and one dog died on the second day of the study). There was a significant positive correlation between MDA concentration (Table 1) and the lethal outcome of the disease (Tau-b 0.309; P 0.005).

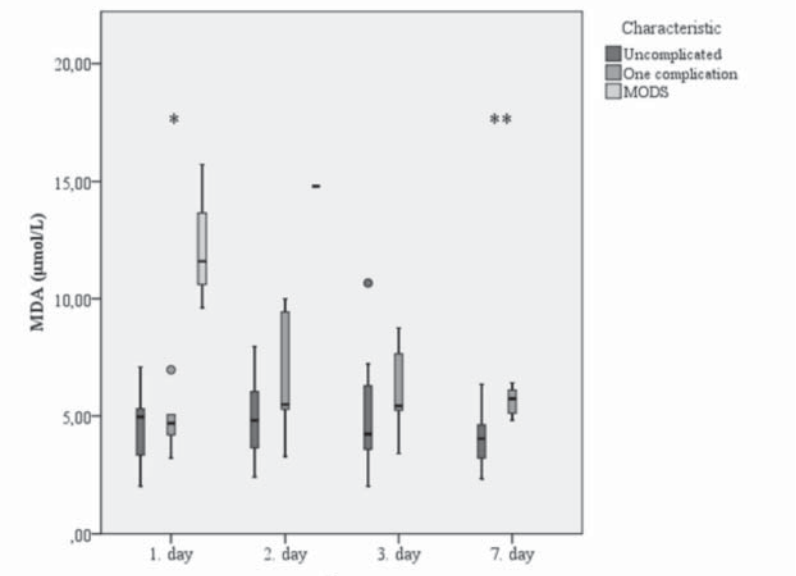

Fig. 2. Malondialdehyde concentration in studied group of dogs infected with *B. canis canis* before (1st day) and after antibabesial treatment (2nd, 3rd and 7th day) of research.

***** The MODS babesiosis group is significantly different than uncomplicated (P = 0.006) as well as one complication babesiosis group (P = 0.025). ****** The one complication babesiosis group is significantly different than the uncomplicated babesiosis group (P 0.003). Plots show the median (dash within box), 25th and 75th percentiles (box), and range (whiskers).

Table 1. Correlations of the investigated parameter with outcome - Tau-b correlation test

Nominal correlation coefficient		MDA (μmol/L)
Lethal outcome	Tau-b	0.309
	P	0.005

Discussion

Canine babesiosis is an emerging, potentially life-threatening hemolytic disease of worldwide significance. Canine babesiosis in Croatia occurs predominantly in its uncomplicated form (BARIĆ RAFAJ et al., 2009; MATIJATKO et al., 2009). In this study the uncomplicated form was present in 73 %, which is consistent with previous studies done in Croatia. Of all the dogs that developed the complicated form of babesiosis, 63 % had single organ dysfunction and 37 % developed MODS. The mortality rate in this study was 10 %, and all the dogs that died had the complicated form of babesiosis with MODS. These results are consistent with previous studies of canine babesiosis in Croatia (MATIJATKO et al., 2009) as well as South African studies of canine babesiosis caused by *B. canis rossi* (COLLETT, 2000; NEL et al., 2004). Various studies on babesiosis caused by *B. canis* throughout Europe demonstrate a wide range of mortality rates, varying from 1.5 % (France) to 20 % (Hungary) (MATHE et al., 2006). The explanation for this could be the presence of a different strain of *B. canis canis* in Croatia. Different strains of *B. canis* and *B. rossi* have been identified and proven to cause different clinical forms of babesiosis with different outcomes (CARCY et al., 2006; MATJILA et al., 2009).

In recent years, many studies have focused on the assessment of the potential role of ROS in the pathogenesis of various parasitic infections. They often indicate that infections caused by various parasites are associated with a significant increase in lipid peroxidation (MURASE et al., 1996; BILDIK et al., 2004; ASRI REZAEI and DALIR-NAGHADEH, 2006; KUMAR et al., 2006; CRNOGAJ et al., 2010). Malondialdehyde is one of the end products of lipid peroxidation and is excreted in the urine, blood, and other body fluids, so it serves as a marker of lipid peroxidation and the presence of oxidative stress (MARKS, 1996).

In the present study, MDA concentrations, on the first day, were significantly increased in dogs with babesiosis in comparison to the control group. Similarly, increases in MDA have also been reported in research conducted by other authors on dogs suffering from *B. canis* (CRNOGAJ et al., 2010) and *B. gibsoni* (OTSUKA et al., 2001; KUMAR et al., 2006; CHAUDHURI et al., 2008). This increase in MDA concentrations might be an indicator that oxidative stress plays a role in the pathogenesis of babesiosis in dogs.

To the best of our knowledge the dynamics of serum MDA concentrations after antibabesial treatment have not been reported previously in canine babesiosis. In this study, the highest concentration of MDA, in affected group of dogs, was on the first day.

Increased concentrations of MDA have been reported in *B. gibsoni* infection (MURASE et al., 1996; OTSUKA et al., 2001; CHAUDHURI et al., 2008), *B. canis* infection (CRNOGAJ et al., 2010) and in mixed infection of *Erlichia canis* and *B. gibsoni* (KUMAR et al., 2006). The higher concentration of MDA in dogs with babesiosis may probably be ascribed to multiplication of babesia parasites (OTSUKA et al., 2001). The lowest concentrations of MDA in this research were found on the seventh day, but they were not significantly different compared to the first day. Concentrations of MDA on the seventh day in group infected with babesiosis were still statistically higher compared to the control group. This result indicates that on the seventh day of this study increased lipid peroxidation is still present in the affected group of dogs.

To our knowledge there is limited data on the influence of oxidative stress in the development of complicated babesiosis and MODS in dogs and other animals suffering from babesiosis or theileriosis (CRNOGAJ et al., 2010). However, there are numerous studies that confirm the involvement of oxidative stress in various diseases in animals, such as liver disease, cardiovascular disease, chronic renal disease, and diseases of the central nervous system (MANDELKER, 2008).

In our study MDA concentrations, on the first day of research, were not significantly different between dogs that developed the uncomplicated form of babesiosis versus dogs with the complicated form, while both groups had significantly higher concentrations of MDA in comparison with the control group. These results are in concordance with the study done on dogs suffering from babesiosis (CRNOGAJ et al., 2010). There was no significant difference, on the first day, in the investigated parameters between dogs in the uncomplicated and single complication groups. Dogs in the MODS group had significantly increased MDA concentrations compared to dogs in the previously mentioned groups. Our results suggest that oxidative stress could contribute to the development of MODS in dogs affected with babesiosis. These results are in accordance with studies done on malaria (KRISHNA et al., 2012).

The highest concentratiosn of MDA in uncomplicated group of dogs were found on the first day of the study, while dogs from the complicated group had the highest concentration of MDA on the second day. On the seventh day the concentration of MDA was statistically higher in the group of dogs with a single complication, compared to the uncomplicated group. These results indicate higher lipid peroxidation and consequently slower recovery in dogs that had developed complications.

Markers of oxidative stress (particularly MDA) are prognostic indicators of the severity of the illness, as well as disease outcome in critically ill people admitted to the intensive care unit of a hospital (MISHRA et al., 2005; MISHRA, 2007). The results of our study demonstrate a significant positive correlation between MDA concentration and the outcome of the disease. Our results are in agreement with the above mentioned research

in human medicine and indicate the potential role of MDA as a prognostic marker of disease severity and outcome in dogs suffering from babesiosis. These results need to be reinforced on a larger number of dogs naturally infected with *B. canis canis.*

Considering the results we may conclude that *B. canis canis* infection in dogs is associated with a high concentration of lipid peroxidation, which indicates the presence of oxidative stress and its possible role in the pathogenesis of the disease. On the seventh day of the study high concentrations of lipid peroxidation are still present in the affected group of dogs. The results indicate that oxidative stress could have a possible causative role in the clinical severity of the disease.

We would like to point out that oxidative stress is a complex process. Therefore, we are aware that, although MDA is a reliable and commonly used biomarker for assessing lipid peroxidation (MOORE and ROBERTS, 1998) and although our results are original, they should be strengthened with more parameters of oxidative stress markers and antioxidants in further investigations. Also, the present results should be strengthened by further investigations into the exact role of oxidative stress, particularly in complicated babesiosis, on larger number of dogs.

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SAŽETAK

Moguća uloga oksidacijskog stresa u patogenezi infekcije uzrokovane parazitima aktivno je područje znanstvenih interesa u posljednjih nekoliko godina. Cilj ovog istraživanja bio je utvrditi promjene u koncentraciji malondialdehida (MDA), najčešće korištenog biomarkera lipidne peroksidacije kod pasa oboljelih od babezioze uzrokovane vrstom *Babesia canis canis*, nakon terapije imidokarb dipropionatom i usporediti ih s koncentracijom MDA u bolesnih pasa prije liječenja i pasa kontrolne skupine. Istraživanje je provedeno na uzorku od 30 pasa oboljelih od babezioze i 25 zdravih pasa (kontrolna skupina). Svim psima određivana je kompletna krvna slika, biokemijski pokazatelji te koncentracija MDA. Krv je izvađena prije terapije - prvi dan (svim psima) te drugi, treći i sedmi dan poslije terapije (bolesni psi i deset zdravih pasa). Primjena imidokarb dipropionata u deset zdravih pasa nije utjecala na koncentraciju MDA te su svi zdravi psi uključeni u kontrolnu skupinu. Prvi dan istraživanja koncentracija MDA bila je značajno viša u skupini bolesnih pasa u odnosu na kontrole ($P<0,001$). Koncentracija MDA padala je prema sedmom danu, međutim i dalje je bila značajno viša nego u kontrolnoj skupini ($P<0,001$). Koncentracija MDA bila je značajno viša prvi dan istraživanja u pasa s jednostavnim ($P<0,001$) odnosno kompliciranim ($P<0,001$) oblikom babezioze u odnosu na kontrolnu skupinu. Sedmi dan istraživanja koncentracija MDA bila je značajno viša u pasa s kompliciranim oblikom babezioze ($P = 0,003$) u odnosu na one s jednostavnim oblikom. Nadalje, u pasa u kojih se razvio sindrom višestrukog zatajenja organa u prvom danu istraživanja, koncentracija MDA bila je značajno viša u usporedbi s onima u kojih je uočena jedna komplikacija ($P = 0,025$), odnosno jednostavni oblik babezioze ($P = 0,006$). Ustanovljena je značajna pozitivna korelacija između koncentracije MDA i ishoda bolesti (Tau-b 0,309; $P = 0,005$). Rezultati ovog istraživanja pokazuju da je sedmi dan istraživanja lipidna peroksidacija i dalje prisutna u pasa oboljelih od babezioze. Također naznačuju da bi oksidacijski stres mogao imati ulogu u težini bolesti.

Ključne riječi: oksidacijski stres, malondialdehid, jednostavni oblik babezioze, kompliciran oblik babezioze