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EFFECTS OF *Ehrlichia canis*, *Anaplasma phagocytophilum /Anaplasma platys* AND *Dirofilaria immitis* INFECTIONS ON OXIDATIVE STRESS AND ANTIOXIDANT BALANCE IN DOGS

B. Dokuzeylül[1*], A. Kırbaş[2], A. Kayar[1], F.M. Kandemir[3], S. Küçükler[4], M.E. Or, and Ł. Adaszek[5]

[1]Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Internal Medicine, 34320 Istanbul, Turkiye
[2]Ataturk University, Faculty of Veterinary Medicine, Department of Internal Medicine, 25240,Turkiye
[3]Aksaray University, Faculty of Medicine, Department of Medical Biochemistry, 68100 Aksaray, Turkiye
[4]Atatürk University, Department of Biochemistry, Faculty of Veterinary Medicine, 25240 Erzurum, Turkiye
[5]University of Life Sciences, Faculty of Veterinary Medicine, Department of Epizootiology and Clinic of Infectious Diseases, 20-612 Lublin, Poland
[*]Corresponding author's email: b9eylul@iuc.tr; bdokuzeylul@gmail.com

ABSTRACT

The aim of the study was to investigate changes in serum oxidant/antioxidant balance in dogs of different breeds and sex, which were infected with *Ehrlichia*, *Anaplasma* or *Dirofilaria immitis*. We also sought to analyze the impact of pathogen species and canine sex on the changes in oxidative stress markers, nitric oxide level, and hematological parameters. A total of 59 dogs of different breeds and sexes were included in the study. Forty-one dogs infected with either *Dirofilaria immitis*, *Anaplasma* or *Ehrlichia* formed the study group, while the control group comprised of 18 healthy dogs with negative test results. Serum nitric oxide (NO), total oxidant capacity (TOC) and total antioxidant capacity (TAC) levels were measured. Oxidative stress index (OSI) was determined by calculating TOC-to-TAC ratio. In dogs with *Ehrlichia* or *Anaplasma* positivity, circulating NO and TOC levels were found to have increased significantly while heartworm infection did not significantly alter TOC levels. A significant decrease in TAC was observed in all animals with positive test results for any pathogen. The results indicated that *Anaplasma*, *Ehrlichia* and *Dirofilaria* infections cause adverse effects on the host cell redox balance in dogs, however, sex has no impact on oxidative stress markers, nitric oxide level, or hematological parameters.

Keywords: *Anaplasma* spp.; *Ehrlichia* spp.; oxidative stress; TAC; TOC.

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INTRODUCTION

Canine ehrlichiosis and anaplasmosis are two important tickborne diseases seen throughout the world which are caused by the Gram-negative intracellular pathogens *Anaplasma* spp. and *Ehrlichia* spp. (Ismail *et al*., 2010; de Caprariis *et al*., 2011). The prevalence of infections with both of these species differs greatly between countries, ranging from 0.2% to 70.5% (Batmaz *et al*., 2001,; Hamel *et al*., 2009; de Caprariis *et al*., 2011). *Ehrlichia canis* is the causative agent of canine monocytic ehrlichiosis and is transmitted by *Rhipicephalus sanguineus*. Three clinicopathological stages of ehrlichiosis have been recognized in dogs: an acute stage, during which dogs exhibit thrombocytopenia and variable clinical signs (lethargy, fever, lymphadenomegaly, and epistaxis); a subacute phase characterized by hyperglobulinemia, thrombocytopenia, and anemia; and a chronic stage in which dogs remain seropositive and can display a number of different clinicopathological findings (thrombocytopenia, lethargy, and pancytopenia) (Fourie *et al*., 2013; Pérez Vera *et al*., 2014; Kaewmongkol *et al*., 2017; Dhliwayo *et al*., 2019; de Paiva Diniz, 2020; Atif *et al*., 2021).

Infections with *A. phagocytophilum*, the causative agent of human granulocytic anaplasmosis, have been increasingly diagnosed in both companion and farm animals (Zynger *et al*., 2009; Dzięgiel *et al*., 2013; Dhliwayo *et al*., 2019; Nicholson *et al*., 2019). This pathogen is described widely in the blood of companion animals (Adaszek *et al*., 2012; Dzięgiel *et al*., 2013), and usually causes an acute infection in dogs, characterized by thrombocytopenia and fever –although subclinical infections have been reported. The main vector carrying *A. phagocytophilum* is the Ixodes tick (Jaenson *et al*., 2012). In direct blood smears, the presence of *Anaplasma* spp. can be demonstrated more easily and more frequently than *Ehrlichia* spp. However, only polymerase

chain reaction (PCR) assays and sequencing are able to discriminate between these pathogens (Kohn *et al*., 2011). The nematode *Dirofilaria immitis* causes canine heartworm disease. In infected animals, the nematode is localized along the pulmonary arteries, in the heart, subcutaneous tissue and muscular connective fasciae (Kramer *et al*., 2008; Wang *et al*., 2014). Although the parasites may be transmitted by ticks and flies, their main vector are mosquitos –with over 70 species capable of transmitting dirofilariasis. Changes in erythrocyte morphology can cause abnormal rheological and respiratory functions as a result of altered elasticity and deformability (Sergeeva *et al*., 2016). This makes erythrocytes ideal for assessing the effects of both exogenous and endogenous factors on an organism-wide scale (Ullah *et al*., 2023).

Except for allergic reactions, and rarely skin and ocular manifestations, clinical findings are seldom observed during the course of *Dirofilaria* infection. However, once the parasite localizes in the pulmonary arteries or the heart, the infection may be fatal (Genchi *et al*., 2012; Petry *et al*., 2015). The diagnosis of Dirofilariosis is based on hematological, serological and echocardiography-based analyses. Reactive oxygen species (ROS) are the main determinants of oxidative stress and are short-lived, unstable molecules (Pereira *et al*., 2015). Oxidative stress may develop if the overproduction of ROS exceeds the neutralization capacity of the antioxidant defense system in cells. Several bacterial, viral and parasitic infections have been linked to oxidative stress (Alberdi *et al*., 2019; Ciftci *et al*., 2021). Since increased ROS disrupts the redox balance, it causes dysfunctions in signaling pathways within the cell, resulting in cell injury (Neer *et al*., 2002). In addition to enzymatic and non-enzymatic antioxidant defense mechanisms, the cell tries to neutralize ROS with thiols or sulfhydryl (–SH) groups (Cakirca *et al*., 2019). *Anaplasma* spp. cause cell damage through the NADPH oxidase-mediated ROS pathway (Alberdi *et al*., 2019). Blood parameters have been demonstrated as reliable biomarkers for evaluating toxicant exposure in living organisms (Hussain *et al*., 2012). The erythrocytes, which are natural targets for free radicals, are susceptible to redox imbalance due to the presence of fatty acids in their membranes, high levels of oxygen, and the presence of hemoglobin within the cell (Ben Saad *et al*., 2014). Furthermore, toxicity may develop in erythrocytes, resulting in deformation, hemolysis, and mitochondrial dysfunction (Zemlyanova *et al*., 2021; Ullah *et al*., 2023).

In a previous study, the effects of the administration of Ca-nanoparticles were investigated in rats, showing development of oxidative stress in tissues. The researchers concluded that nanoparticle administration induced oxidative stress, altered blood biomarkers, and caused histopathological changes in several organs (Ullah *et al*., 2023). Oxidative stress can be assessed by measuring the biomarkers of free radical damage in the biological system and alterations in antioxidant defense mechanisms. This is particularly associated with the fact that cellular structures may be damaged by oxidative stress (Akram *et al*., 2022; Al-Saeed *et al*., 2023). While *Dirofilaria* worms block innate immunity, *Ehrlichia* increases ROS production through activation of cell-mediated immune response, damaging the host cell (Ciftci *et al*., 2011; Kohn *et al*., 2011; Rubio *et al*., 2017). It has been claimed that oxidative stress may be one of the factors causing cellular injury during the course of infections with *Anaplasma spp.*, *Ehrlichia* spp. and *Dirofilaria immitis*. In previous studies, the effects of these three pathogens on oxidative stress markers and hematological parameters were investigated (Ciftci *et al*., 2011; Kohn *et al*., 2011; Rubio *et al*., 2017; Alberdi *et al*., 2019). However, data is limited regarding the impact of sex on the production of hematological and oxidative stress markers in dogs.

The aim of this study was to investigate changes in serum oxidant/antioxidant balance in dogs of different breed and sex suffering from ehrlichiosis, anaplasmosis and dirofilariasis. Using a general linear regression model, the impact of the infections on oxidative stress markers, nitric oxide and hematological parameters were investigated with respect to pathogen species and dog sex. Instead of measuring individual oxidant and antioxidant markers, total oxidant capacity (TOC), total antioxidant capacity (TAC), and oxidative stress index (TOC/TAC=OSI) values were calculated.

MATERIALS AND METHODS

Animal selection, grouping, ethical statement and consent to participate: The study was carried out between March 2020 and February 2022 in accordance with the standards set by the Council of Europe on the protection of animals used for scientific purposes (2010/63/EU). Consent forms were obtained from pet owners. The study was carried out on dogs admitted to the Department of Internal Medicine, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa. A total of 59 dogs (34 males and 25 females) of different breeds (American Cocker, Anatolian Shepherd, Australian Shepherd, Bulldog, English Setter, German Shepherd, Golden Retriever, Irish Setter, Pointer, Mixed Breed, Siberian Husky, White Coated Terrier, Yorkshire Terrier) were included in the study. All participants were selected from dogs older than 1 years of age. Forty-one dogs infected with either *Dirofilaria immitis*, *Anaplasma* or *Ehrlichia* formed the study group, and the control group comprised of 18 healthy dogs with negative test results. The dogs included in the study group had been referred with varying clinical symptoms including lethargy, anorexia, fever, abdominal distension, epistaxis and exercise intolerance. The control group was

composed of dogs that had been admitted for routine check-up and vaccinations.

Animals residing in regions with high tick prevalence (regardless of the detection of tick parasitism), animals with known tick infestation, those with anemia, leukopenia or thrombocytopenia, and subjects with clinical symptoms compatible with anaplasmosis, ehrlichiosis and/or heartworm disease were tested for the three pathogens. Eight dogs were Dirofilaria-infected, 12 dogs had antibodies for *Anaplasma*, and 21 were positive for *Ehrlichia.* The control group consisted of 18 adult dogs with unremarkable findings in complete blood count and serum biochemical analyses who were negative for these three pathogens as evidences by serological testing. These 18 healthy controls were further subdivided into three subgroups, each comprising 6 animals. These three healthy subgroups were separately compared to each group of pathogen-positive dogs.

Ehrlichiosis, anaplasmosis and dirofilariasis screening with rapid ELISA test: Rapid diagnostic blood ELISA test kits (SNAP 4Dx®, IDEXX Laboratories, Westbrook, ME, USA) were used for the diagnosis of *Ehrlichia canis*/*Ehrlichia ewingii*, *Anaplasma phagocytophilum*/*Anaplasma platys*, Lyme disease and Heartworm disease. The test detects *Dirofilaria immitis* antigens and antibodies for *Ehrlichia canis/Ehrlichia ewingii, Anaplasma phagocytophilum*/*Anaplasma platys* and *Borrelia burgdorferi.* The dogs infected with these pathogens yielded positive results for either *Dirofilaria* antigens or antibodies against *Anaplasma* spp./*Ehrlichia* spp. Antibodies against *Ehrlichia* and *Anaplasma* were detected in seven dogs. All dogs in the control group were negative for *Dirofilaria* antigen, and antibodies against *Anaplasma* and *Ehrlichia.*

Collection of blood samples: Blood samples were obtained from the jugular vein. The centrifugation of venous blood samples was carried out at 3000 rpm for 10 minutes, and aliquoted blood samples were stored at -20°C until analyses. Hematological parameters were analyzed in a hematology analyzer device (ProCyte Dx Haematology Analyzer, Idexx). Analysis of biochemical parameters [Blood urea nitrogen (BUN), Creatinine (CRE) and Alanine-Amino Transferase (ALT)] was performed with a biochemistry autoanalyzer (Catalyst one Chemistry Analyzer, Idexx). Plasma glucose levels were measured by the Glucose oxidase method. Plasma troponin levels were measured with a fully automated assay (Roche Diagnostics).

TOC, TAC and OSI Analyses: TAC and TOC levels were measured in an autoanalyzer by using TAC and TOC kits (Rel Assay, Mega Medicine Industry, Gaziantep, Türkiye). Briefly, the oxidative reaction induced by hydroxyl radicals was blocked by antioxidant molecules in the mixture to measure TAC, which was calculated by measuring the color intensity of the mixture. Plasma oxidants present in the mixture convert ferrous ions to ferric ions. Glycerol is added to the medium to further strengthen the oxidation reaction. Color intensity measured via spectrophotometry was used to measure TOC, as ferric ions form an orange color with xylenol. TAC was presented in mmolTrolox Equivalent/L units, while TOC was presented with μmol H_2O_2 Equivalent/L units. OSI value was calculated as the TOC-to-TAC ratio (OSI = TOC/TAC), which was presented with percentage values.

Total NO analysis: Plasma total NO level was measured using an NO detection kit (Enzo Life Science). This measurement was made according to the colorimetric evaluation of nitrite, which is the product of the Griess reaction (enzymatic conversion of nitrate to nitrite).

Statistical Analysis: Statistical analysis was performed using Statistical Package for the Social Sciences for Windows version 13 (SPSS Inc., Chicago, IL, USA). Normality of distribution in continuous variables was assessed by using the Shapiro–Wilk test. Comparative analyses were performed with the Student's t-test for normally distributed continuous variables, chi-square tests for categorical variables, and the Mann–Whitney U test for non-normally distributed continuous variables. Continuous variables were presented as mean ± standard deviation and categorical variables were expressed in counts and percentages (*n*, %). The General Linear Model analysis method was used to determine the effects of dirofilariosis (presence or absence), anaplasmosis (presence or absence), ehrlichiosis (presence or absence) and animal sex (male or female) on biochemical and hematological parameters. In animals infected with both *Ehrlichia* and *Anaplasma*, TAC, TOC, OSI and NO were evaluated by subgroup analysis.

RESULTS

The different dog breeds included in the study population (infected and healthy controls) are described in ***Table 1***. Body weight (BW) ranged between 9 and 37 kg, and the average weight was 18.6±8.20 kg in the experimental group and 17.3±9.40 kg in the control group. There was no difference between the two groups regarding BW ($p = 0.20$). Mean age was 3.55±1.24 years in the experimental group and 3.12±1.56 years in the control group. The mean age of the two groups was similar ($p = 0.67$).

In dogs infected with Dirofilaria, Anaplasma or Ehrlichia, significant differences were detected in at least one of the following parameters: NO, TAC, TOC, HGB, PLT, MCHC or glucose. Differences in NO, TAC, TOC, HGB, PLT, MCHC and glucose levels among the study groups are presented in ***Table 2***.

On the other hand, the serum levels of Troponin I, RBC, HCT, WBC, MCV, MCH, ALT, BUN, and CRE were unassociated with the infection status (Anaplasma, Ehrlichia or Dirofilaria). No significant differences were observed in serum troponin I, RBC, HCT, WBC, MCV, MCH, ALT, BUN and CRE levels between the experimental and control groups. Dogs with positive serology results for *Ehrlichia* or *Anaplasma* had significantly higher NO levels ($p = 0.010$ and $p = 0.017$, respectively), while infection with *Dirofilaria immitis* did not cause a significant change in serum NO levels ($p = 0.243$). In dogs with positive serology for *Anaplasma* and *Ehrlichia*, blood TOC levels were significantly higher ($p = 0.011$ and $p = 0.017$, respectively), while *Dirofilaria* infection did not cause a significant difference in TOC ($p = 0.072$). Significantly decreased TAC levels were detected in dogs with *Dirofilaria* and *Anaplasma* ($p = 0.008$ and $p = 0.019$, respectively). OSI values (TOC/TAC) were: 1.84, 1.84 and 1.92, respectively in *Dirofilaria*, *Anaplasma*, and *Ehrlichia* positive animals. These values were found to be significantly higher among dogs in the experimental group compared to the control group (1.44, 1.42 and 1.37, respectively, $p < 0.05$ for all). TAC, TOC, OSI and NO values in animals with positive test results for *Ehrlichia* and *Anaplasma* were found to be similar to controls.

HGB level, PLT total count and MCHC value were significantly decreased only in dogs with antibodies against *Anaplasma* ($p = 0.037$, $p = 0.014$, and $p = 0.041$, respectively). The presence of *Dirofilaria* or *Anaplasma* did not cause a significant change in serum glucose. On the other hand, in dogs with antibody positivity against *Ehrlichia*, a significant decrease in serum glucose level was observed ($p = 0.041$).

Canine species and sex (male or female) were unassociated with oxidative stress balance markers, hematological parameters or biochemical parameters, as determined by the general linear model.

Table 1. The breeds and number of dogs included the study and control group.

Dog Breeds	Study Group	Control Group
Golden Retriever	6	2
Siberian Husky	3	2
American Cocker	4	1
German Shepherd	7	2
Anatolian Shepherd	4	-
English Setter	2	2
White Coated Terrier	5	-
Mixed Breed	6	7
Irish Setter	2	-
Bulldog	1	-
Pointer	1	-
Australian Shepherd	-	1
Yorkshire Terrier	-	1

Table 2. Comparison of oxidant, antioxidant, biochemical and hematological parameters of dogs with positive snap test for Ehrlichia, Anaplasma and/or Dirofilaria and healthy controls.

Parameters	Dirofilaria + (n=8)	Control (n=6)	*p*	Anaplasma + (n=12)*	Control (n=6)	*P*	Ehrlichia + (n=21)*	Control (n=6)	*p*
NO (µmol/L)	247.1 ± 31.0	208.4 ± 14.9	0.243	316.8 ± 27.2	138.6 ± 17.8	0.017**	258.1 ± 21.7	197.3 ± 21.7	0.010**
TAC (mmol/L)	1.10 ± 0.06	1.29 ± 0.03	0.008**	1.13 ± 0.05	1.26 ± 0.03	0.019**	1.09 ± 0.04	1.30 ± 0.04	0.041*
TOC (µmol/L)	2.03 ± 0.09	1.86 ± 0.04	0.072	2.09 ± 0.07	1.80 ± 0.05	0.011**	2.10 ± 0.06	1.79 ± 0.06	0.017**
Glucose (mg/dl)	104.8 ± 6.12	105.2 ± 4.61	0.967	99.0 ± 5.58	111.0 ± 4.89	0.120	96.6 ± 4.81	113.3 ± 5.96	0.041*
Troponin-I (µg/L)	0.18 ± 0.15	0.49 ± 2.39	0.769	0.83 ± 2.52	0.65 ± 2,36	0.134	0.63 ± 1.89	0.74 ± 1.43	0.253
RBC(x10^6/µL)	4.84 ± 1.58	4.76 ± 0.57	0.879	4.12 ± 1.46	5.22 ± 2,11	0,164	4.83 ± 1.44	3.78 ± 1.36	0,354
HGB(g/dl)	9.46 ± 1.16	10.1 ± 0.87	0.651	8.28 ± 1.02	11.3 ± 0.93	0.037*	9.78 ± 0.89	9.83 ± 1.14	0.969
HCT (%)	33.25 ± 11.13	35.1 ± 2.46	0.919	28 ± 8.28	26 ± 6.34	0.213	33.11 ± 9.32	35.43 ± 7.24	0,763
WBC (x10^3/µL)	14.21 ± 7.17	10.03 ± 4.26	0.125	16.82 ± 11.9	11.23 ± 6.92	0.058	13.61 ± 10.34	9.56 ± 4.49	0.147
PLT (x10^3/µL)	258.9 ± 59.2	220.0 ± 44.2	0.616	141.9 ± 52.2	337.0 ± 47.3	0.014**	180.2 ± 45.3	298.7 ± 57.8	0.131
MCV (µm^3)	68.5 ± 2.07	58.54 ± 2.28	0.724	69.75 ± 8.24	67.87 ± 7.98	0.443	69.67 ± 5.2	67.98 ± 4.46	0,155
MCH (pg)	21 ± 0.93	26.51 ± 1.78	0.870	21.08 ± 2.27	22.05 ± 2.75	0.526	21.39 ± 1.42	24.75 ± 1.58	0,326
MCHC (g/dl)	30.4 ± 0.51	31.0 ± 0.38	0.357	30.1 ± 0.45	31.3 ± 0.41	0.041*	30.6 ± 0.39	30.8 ± 0.50	0.718
ALT(IU/L)	70.38 ± 37.07	65.24 ± 29.75	0.629	61.91 ± 57.79	55.83 ± 49.98	0.112	51.82 ± 44.9	50.65 ± 42.65	0.139
AST (IU/L)	45.75 ± 13.48	52.45 ± 14.43	0.723	45.73 ± 27.06	43.65 ± 25.09	0.298	50.71 ± 28.37	52.28 ± 25.78	0.267
BUN (mg/dl)	40.21 ± 28.93	42.34 ± 24.55	0.428	35.27 ± 17.01	34.18 ± 16.93	0.143	37.29 ± 24.26	34.93 ± 22.24	0.194
Creatinine (mg/dl)	0.88 ± 0.41	0.76 ± 0.58	0.549	0.8 ± 0.28	0.63 ± 0.32	0.157	0.89 ± 0.4	0.56 ± 0.32	0.125

DISCUSSION

ROS are by-products of normal cell activity which are produced in many cellular processes, and they play a major role in signaling pathways (Snezhkina *et al*., 2019). Controlled production of mitochondria-derived ROS in humans and animals ensures the maintenance of the redox balance and enables the cell to continue its metabolic functions. An imbalance between oxidant and antioxidant compounds causes an increase in free radicals, and ROS cause tissue damage in a variety of pathologies, such as parasitic disease (Baldissera *et al*., 2015). Overproduction of ROS causes acute and/or chronic cell damage by disrupting the intracellular oxidant/antioxidant balance. Chronic infections are one of the most important factors leading to elevated ROS production in animals (Celi *et al*., 2010). Canine ehrlichiosis, anaplasmosis and dirofilariosis are the most common vector-borne diseases that trigger oxidative stress production in host cells (Neer *et al*., 2002; Rubio *et al*., 2017; Alberdi *et al*., 2019; Cakirca *et al*., 2019). Cells infected by the pathogen attempt to protect cellular integrity by activating enzymatic or non-enzymatic antioxidant mechanisms, neutralizing the increase in ROS. If free radical production is not quenched by antioxidants, many signaling pathways that control vital cellular functions are disrupted, leading to oxidative damage (Fang *et al*., 2002; Schieber *et al*., 2014).

Changes in blood parameters in the course of ehrlichiosis, anaplasmosis and dirofilariosis may be a result of blood cell injury mediated by oxidative stress. Non-regenerative anemia and thrombocytopenia alongside hyperglobulinemia, hypoalbuminemia and low albumin-globulin ratio are among the biochemical abnormalities frequently encountered in CME (Parashar *et al*., 2016). Significantly increased TOC in the serum of dogs infected with *Anaplasma* or *Ehrlichia* is an important indication of the presence of oxidative cell damage. Similarly, a significant decrease in TAC levels suggests that the oxidant/antioxidant balance is impaired and the defense mechanisms of the cell are weakened. The coexistence of both of these pathogens was not found to cause an additive increase in TOC. In such cases, a high pathogen burden may limit oxidative response due to immunosuppression. However, the fact that animals infected with these three pathogens may live for many years without showing clinical symptoms of infection suggests that the antioxidant defense systems of the host cells are quite successful in maintaining redox balance (Sainz *et al*., 2015).

The normal serum TOC levels in *Dirofilaria*-positive dogs are indicative of the fact that the enzymatic and non-enzymatic free radical scavenger activities in these animals are sufficient. The activity of free radicals such as thiobarbituric acid reactive substances and ferrous oxidation (-xylenol orange) have been reported to increase significantly during the course of canine monocytic ehrlichiosis (Rubio *et al*., 2017). The same authors also found that TAC, cupric-reducing antioxidant capacity, and ferric-reducing capacity decreased in dogs infected with *Ehrlichia* (Rubio *et al*., 2017). Our results are similar to the literature and show that in the course of infection with *Anaplasma, Ehrlichia* and *Dirofilaria*, increased production of ROS occurs in target cells, while free radical quenchers and antioxidant capacity decreases. Disruption of the redox balance may allow the infection to progress into the subclinical or chronic stage and may lead to prolonged pathogen survival in host cells –without clinical symptoms.

Clinical and laboratory findings in canine vector-borne diseases are unspecific. The type of pathogen, the immune response of the host, and other concomitant infections are the main determinants of overt clinical and laboratory findings (Sainz *et al*., 2015). None of the dogs included in our study had fever. Snap tests were performed according to the findings recorded during clinical examination and complete blood count results.

In the hematological tests performed on *Anaplasma*-infected animals, we found that PLT counts were also significantly lower compared to healthy controls (in addition to HGB and MCHC). In *Ehrlichia*- or *Dirofilaria*- positive animals, the levels of HGB, PLT and MCHC were similar to the control group. There may be more than one reason explaining the differences (or lack thereof) in hematological parameters between the studied groups. First of all, the dog's sex and type of infection may have an impact on the laboratory findings. Although *Ehrlichia* can infect all dog breeds, disease severity may differ in particular breeds or cases, likely as a result of variations in cell-mediated immune response (Mylonakis and Theodorou, 2017). It has been suggested that there is no link between *Anaplasma* infection and the breed, age, or sex of the animal (Kohn *et al*., 2011). In the linear regression analysis, we similarly showed that the dog breed and sex had no effect on laboratory parameters. It is also possible that the simultaneous presence of other vector-borne pathogens may explain the differences in laboratory findings between studied groups. The presence of concurrent endosymbiont bacteria, especially in dirophilarial infection, may cause changes in clinical and laboratory findings (Genchi *et al*., 2012). Since we did not evaluate pathogens other than the six pathogens in the rapid test kit, we cannot draw definitive conclusions concerning this issue.

The second possible reason for differences in laboratory findings is the immune response of the animal. The clinical course of ehrlichiosis may be acute, subclinical, or chronic at detection (McClure *et al*., 2010). It is often not possible to distinguish the exact phase of disease. Although the main reason for the progression of the infection to the chronic phase is unknown, this phase is characterized by bone marrow

hypoplasia and severe pancytopenia (Mylonakis *et al*., 2004). *Anaplasma*-infected dogs develop a self-limiting acute febrile disease. Most dogs are clinically healthy even in the presence of antibodies for *Anaplasma* (Egenvall *et al*., 2000). Although *Ehrlichia* infections cause a relatively severe clinical course compared to *Anaplasma*, the symptoms are often similar in both diseases (Little, 2010). Notwithstanding the fact that most laboratory findings related to canine ehrlichiosis or anaplasmosis are non-specific, low platelet count is the most common finding. Increased ROS production may be the reason for the reduction in hematological parameters. Despite the increase in TOC levels among both *Anaplasma*- and *Ehrlichia*-positive groups in our study, decreased HGB and platelet count were observed only among *Anaplasma*-positive animals, suggesting that parameters other than free radicals also affect hematological parameters. Detection of diffuse splenomegaly in *Anaplasma*-infected animals indicates that these pathogens cause immune-mediated thrombocytopenia or anemia, regardless of ROS levels (Kohn *et al*., 2011). It is well established that each vector-borne pathogen can demonstrate host-specific cell tropism. For example, ticks increase ROS production thereby limiting *Anaplasma* infection, while anaplasmosis reduces ROS production in blood cells, allowing them to settle in the host (Alberdi *et al*., 2019). Since the immune response of each animal to the discussed pathogens will be different, the resultant oxidative damage to the target cells will also likely vary between subjects. *Anaplasma* inhibits the production of NADPH oxidase-mediated ROS in the target cell, and thus, platelets may be excessively destroyed due to exposure to oxidative damage (Alberdi *et al*., 2019). On the other hand, *Dirofilaria* blocks or suppresses innate immunity in order to spare itself from free radical damage, while *Ehrlichia* has evolved an avoidance mechanism that blocks cell-mediated immune response (Kohn *et al*., 2011; Rubio *et al*., 2017, Ciftci *et al*., 2021).

The current study has found lower serum glucose levels in *Ehrlichia*-positive dogs compared to healthy controls; whereas, *Anaplasma* or *Dirofilaria* infections did not cause a significant change in serum glucose. Since both *Ehrlichia* and *Anaplasma* are obligate intracellular rickettsial pathogens, it is not possible to test the relationship between glucose level and infection with these two pathogens *in vitro*, at least by routine means. In a recent *in vitro* study, the addition of glucose-6-phosphate to the Axenic medium caused increased protein and DNA synthesis in both *Ehrlichia* and *Anaplasma*. Deficiencies in the adrenal and thyroid glands have opposing effects of catabolism and anabolism in Canine monocytic ehrlichiosis. It seems that the progressive wasting in ehrlichiosis may involve a multiplicity of factors. Ehrlichiosis (with or without babesiosis) is a catabolic disease, and thus, may be responsible for disturbed thyroid metabolism and altered secretion of the endocrine pancreas and adrenals. These relationships could lead to changes in glucose levels (Kumar *et al*., 2006). Taken together with our results, these mechanisms provide support for the role of glucose (and its metabolism) in the life cycle of *Anaplasma* and *Ehrlichia* (Zhang *et al*., 2021). We found that serum NO level was significantly increased in *Ehrlichia*- or *Anaplasma*-positive animals compared to healthy controls. However, being infected with both pathogens was not found to result in a significant difference in serum NO levels.

Dirofilaria infection did not cause a significant change in NO levels. NO is one of the antimicrobial molecules used by macrophages in their disposal of intracellular pathogens (Das *et al*., 2010). NO stimulates innate and cellular immunity against bacterial pathogens more rapidly than ROS (Morris *et al*., 2013). This molecule is produced by infected cells, which suggests that the NO elevation during infection with these two pathogens may be associated with the activation of the NO-mediated antimicrobial pathway.

Dirofilaria immitis is a nematode which primarily localizes in the pulmonary arteries of infected dogs, ultimately causing proliferative endarteritis which narrows the lumen and damages the endothelium and cardiomyocytes (McCall *et al*., 2008). Dogs with heartworm infection have been reported to have elevated troponin I levels (Lee *et al*., 2020). Our analyses showed that none of the examined pathogens caused a significant difference in serum troponin I levels. Troponins are structural elements of the contractile cardiac muscle and are responsible for the interaction between actin and myosin. There are three different troponin subunits: Troponin C, Troponin I and Troponin T (Panteghini *et al*., 2008). Within the first 4 hours after cardiomyocyte injury, troponins are released to the circulation until they are cleared within 5 to 20 days, depending on the severity of muscle injury. Normal troponin I levels in heartworm-positive animals indicate that the contractile apparatus of heart muscle was not damaged, or that troponins had been cleared from the circulation by sampling time. Because none of the dogs used in the study showed acute symptoms of the illness, we can suspect that the infection was in a chronic phase (Katus *et al*., 1989; Park *et al*., 2017).

Despite the limited number of dogs and the fact that data were drawn from rapid diagnostic kits, our study may provide valuable data for future studies, especially since we report a comprehensive set of parameters. Furthermore, this study has analyzed the effects of three different pathogens on hematological, biochemical and oxidative stress parameters, which were examined with respect to various other parameters. Our findings suggest that oxidative stress may play a central role in the pathophysiology of various canine vector-borne diseases.

***Availability of Data and Materials:** The dataset generated during the current study is available from the corresponding author (B. Dokuzeylul) on reasonable request.

Ethical Statement: The study was carried out in accordance with the local ethical committee of Istanbul University-Cerrahpasa, Faculty of Veterinary Medicine on the protection of animals used for a scientific purpose (2022/33). Consent forms were also obtained from the patient owners. The study was carried out on dogs that were referred to Department of Internal Medicine, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa.

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Authors' Contributions: BD, AK and MEO designed the study. BD and AK collected the samples. AK, FMK and SK performed the experiments. BD collected data. BD, MEO and LA analyzed data. BD wrote the manuscript. All authors read the manuscript. All authors interpreted the data, critically revised the manuscript and approved the final version.

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