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Evaluation of Oxidative Status and Inflammatory Changes in Naturally Occurring Canine Visceral Leishmaniasis

Tünay Kontaş Aşkar[1,*], Şinasi Aşkar[1], Olga Büyükleblebici[2] and Murat Güzel[3]

[1]*Department of Nutrition and Dietetic, Faculty of Health Sciences, University of Karatekin, 18200, Çankırı, Turkey*

[2]*Department of Biochemistry, Faculty of Veterinary, University of Aksaray, Aksaray, Turkey*

[3]*Department of Internal Medicine, Faculty of Veterinary, University of Ondokuz Mayıs, Samsun, Turkey*

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TKA and MG designed the study, collected and analysed the data and wrote the article. SA and OB helped in collection and analysis of data and article writing.

Key words
Dog, Inflammatory changes, Leishmaniasis, Oxidative stress.

ABSTRACT

The aim of the study is to determine the oxidative stress and inflammatory changes in naturally occurring canine visceral leishmaniasis (CVL). A total of 30 dogs, comprising 10 clinically healthy and 20 dogs with leishmaniasis were enrolled in this study. The diagnosis of canine visceral leishmaniasis was performed by the immune fluorescence antibody test with antibody titter ≥1:128 and lymph node smear examination. In blood samples of dogs with visceral leishmaniasis, levels of malondialdehyde, total antioxidant capacity, glutathione, nitric oxide, myeloperoxidase, adenosine deaminase and cytokines (TNF-alpha, IL-1β) were determined and compared with the healthy controls. Significantly high levels of plasma malondialdehyde, nitric oxide, myeloperoxidase, adenosine deaminase and cytokines and significantly low levels in total antioxidant capacity and glutathione levels were found in dogs with leishmaniasis when compared with the healthy controls. According to the result of this study, oxidative stress and inflammatory changes occur in CVL. And this is the first report for myeloperoxidase (MPO) activity in CVL. Therefore investigation of this enzyme activity in dogs with leishmaniasis may be used for the diagnosis and inflammatory changes in dogs with VL.

INTRODUCTION

Visceral Leishmaniasis (VL) is a zoonosis in the Mediterranean basin which is very difficult to treat both humans and dogs. VL present in 88 countries on four continents, caused 2.4 million disability-adjusted life years (DALYs) and 59,000 death in 2001 (Alberola *et al*., 2004). The dog is the principal host for VL and in Turkey the infection caused by *Leishmania infantum* (Ozensoy *et al*., 1998).

Macrophages, neutrophils and other phagocytic cells are important compounds of host defense against parasites. These phagocytic inflammatory cells can produce highly toxic molecules such as reactive oxygen and nitrogen species (ROS and RNS) including superoxide radicals (O_2), hydrogen peroxide (H_2O), hydroxyl radicals (OH), nitric oxide (NO) and proinflammatory cytokines (Bogdan *et al*., 2000; Abdel-Maksoud *et al*., 2017). Nitric oxide is a significant cytotoxic and cytostatic tool for diverse cellular parasites with oxidative and prooxidative properties in inflammatory diseases. It has been shown that the interaction between NO and myeloperoxidase (MPO) could have a profound effect on the toxic capacity of neutrophils (Brunet, 2001; Knaapen *et al*., 2005).

Myeloperoxidase is an oxidative enzyme with antimicrobial activity that uses H_2O_2 to produce hypochloric acid (a potent cytotoxic compound) and other toxic substances in neutrophil phagolysosomes. Therefore, MPO is abundant in cytoplasmic granules of neutrophils, and 40-70% of H_2O_2 derived from neutrophils is consumed by MPO (Garça *et al*., 2013). Adenosine deaminase (ADA) is a purine catabolic enzyme with the highest concentration of lymphoid tissue in mammalian tissue. Detection of ADA activity in biological fluids is very useful for diagnosing diseases in which many pathological conditions and immune response are mediated by the cell (Kontaş and Salmanoğlu, 2006). Although the inflammatory and oxidative changes induced by *Leishmania infantum* has been the subject to many investigations (Pinelli *et al*., 1994; Erel *et al*., 1999; Heidarpour *et al*., 2012; Serarslan *et al*., 2005) the mechanisms that underlie host resistance and pathogenesis in CVL are not entirely understood. Therefore, we aimed to determine the inflammatory and oxidative changes in in naturally occurring CVL model.

* Corresponding author: tunaykontas@yahoo.com
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MATERIALS AND METHODS

Animals and clinical examination

In this study, outdoor dogs (n=30) were enrolled in the study. The animals were between 2-6 years old age and 15-35 kg body weight in different breed and both of sex. For all dogs, the clinical history and physical examinations were performed. These dogs with two or more obvious clinical signs of leishmaniosis such as weight loss (16/20), lymphadenopathy (8/20), fever, epistaxis (4/20), fatigue, conjunctivitis (14/20), dermatitis, hair loss, mouth and skin ulcers, onychogryphosis. After clinical examination, the animal with two or more obvious symptoms of the disease was considered as clinical positive and investigated further parasitological analysis to VL. In addition to, suspected or clinical positive animals were evaluated both serological and lymph node smear examination.

Parasitological analysis

Lymph node aspiration

Popliteal lymph node aspirates were taken from the dogs with extended lymph nodes from the clinical positive and negative animals according to the clinical history and physical examinations. After aspiration and fixing in methanol, prepared smears were stained with Giemsa 10% for 10 min at room temperature and examined with light microscope for the presence of amastigotes. Parasitological examination was regarded as positive based on smear positivity.

Immune fluorescence test (IFAT)

The diagnosis of CVL was performed by the immune fluorescence test with antibody titter ≥1:128 and lymph node smear examination. Briefly, the in-house antigen consisted of promastigotes of local *L. infantum* zymodeme MON-1 was used in IFAT. Following incubation at 37°C for 30 min slides were washed and stained with FITC-labeled anti-dog IgG conjugate (Sigma, A9042) for dog sera. Titers ≥1:128 were considered as seropositive (Abranches *et al*., 1991).

Biochemical analysis

Blood samples were collected from dogs and added to lithium heparin and silicone gel-coated test tubes. Serum/plasma samples were separated by centrifugation at 2500 rpm for 10 min at room temperature and kept at -80°C until processing. Blood samples were analyzed for the changes in malondialdehyde (MDA), total antioxidant capacity (TAC), glutathione (GSH), NO and cytokines (TNF-α an IL-1β) levels, in addition to MPO and ADA enzyme activities.

MDA levels in the plasma samples were defined by a formerly defined method (Yoshioka *et al*., 1979), and reduced GSH levels were determined by the method of Beutler *et al*. (1963). Plasma TAC levels were measured by using Rel Assay Diagnostics kit (Mega Tıp, Turkey). This method was based upon the bleaching of the distinct color of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation via the action of antioxidants. The production of nitric oxide (NO) was determined indirectly by measuring the nitrite levels based on Griess reaction (Cortas and Wakid, 1990). MPO activity was measured using the method of Kruidenier *et al*. (2003). Serum samples were incubated for 30 min with 0.5 % hexadecyltrimethylammonium bromide (HTAB) solution (pH 5.5) and 0.026 % ortho-dianisidinedihydrochloride plus 0.018 % H_2O_2. The reaction was specifically confirmed by sodium azide (0.1 mM). MPO activity was expressed as U/L. Serum total ADA activity was determined with Giusti (1974) method. It is a colorimetric manual method based on the principle of measuring absorbance of the coloredindophenole complex at 628 nm. Canine interleukin-1β (Eastbiopharm, Cat No. CK-E90800), and canine tumor necrosis factor-α (Eastbiopharm, Cat No. CK-E90806) levels were measured using the ELISA method. Measurable sensitivity of IL-1β was 0.1 pg/mL, and the test interval of IL-1β level was 0.2 pg/mL and 50 pg/mL, measurable sensitivity of TNF-α is 0.01 ng/L and test interval of TNF-α level was 0,02 ng/L and 8 ng/L.

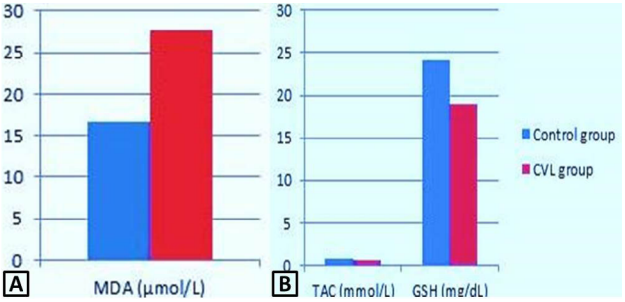

Fig. 1. **A,** changes in MDA levels in dogs with VL and healthy controls. There is a significant increase in CVL group (P<0.001) when compared with the control group. MDA: malondialdehyde, CVL: canine visceral leishmaniasis; **B,** changes in antioxidant defense in dogs with VL and healthy controls. In CVL group, there is a significant reduce in TAC (P<0.05) and GSH (P<0,01) levels when compared with the control group. TAC, total antioxidant capacity; GSH, glutathione; CVL, canine visceral leishmaniasis.

Statistical analysis

Data for biochemical analyses are presented as mean±SD. Statistical analysis was performed using Student's *t*-test; P values <0.05 were considered statistically

significant. The correlation of the MDA concentration with TAC and GSH concentrations, besides the correlation of TNF-α concentration with NO, MPO, ADA, and IL-1β concentrations were analyzed using Pearson's rank correlation coefficient.

RESULTS

In the study, the clinical status of the dogs was carefully assessed, and leishmaniasis dogs showed two or more open clinical findings of leishmaniasis.

The results of oxidative stress and antioxidant status parameters of leishmaniasis and healthy control group dogs are presented in Figure 1. We observed a significant increase in MDA (P <0.001) levels in the CVL group (Fig. 1); and as seen in Figure 1B, there is a significant decrease in TAC (P<0.05) and GSH (P<0.01) levels in CVL group when compared with the control group.

The correlations of MDA level with TAC and GSH levels were analyzed in this study. By using Pearson's rank correlation coefficient, a potent correlation was seen with TAC level (r=-0.618, P<0.05), but there was a low correlation with GSH level (r=-0.69, P<0.001). The negative correlations of MDA level with TAC, and GSH is shown in Figure 2A and B.

Whereas, the levels of NO, MPO, ADA, and cytokines in the dogs with VL and the healthy controls are shown in Table I. NO and TNF-α, IL-1β levels were found significantly higher (P<0.01, P<0.001, and P<0.001, respectively) in CVL in comparison to healthy animals. Serum enzyme activities of ADA and MPO in the dogs with VL were found significantly higher (P<0.001) than that of the healthy animals.

Table I.- Serum cytokine, NO, MPO and ADA levels in dogs with VL and healthy controls.

	Control group (n=10)	CVL group (n=20)	P
NO (µmol/L)	20.87±1.01[a]	29.37±2.62[b]	P<0.01
MPO (ng/mL)	12.02±3.47[a]	24.16±8.59[b]	P<0.001
ADA (IU/L)	5.24±0.24[a]	8.24±0.26[b]	P<0.05
IL-1ß pg/mL	15.24±1.87[a]	27.8±5.12[b]	P<0.001
TNF-α ng/L	0.39±0.83[a]	3.12±0.71[b]	P<0.001

*Data are expressed as mean±standart error of the mean values; [a,b]differences are statistically significant in groups marked with different letters in the same row (P<0.05). NO, nitric oxide; MPO, miyeloperoxidase; ADA, adenosine deaminase; IL-1β, ınterleukine-1β; TNF-α, tumour necrosis factor- α.

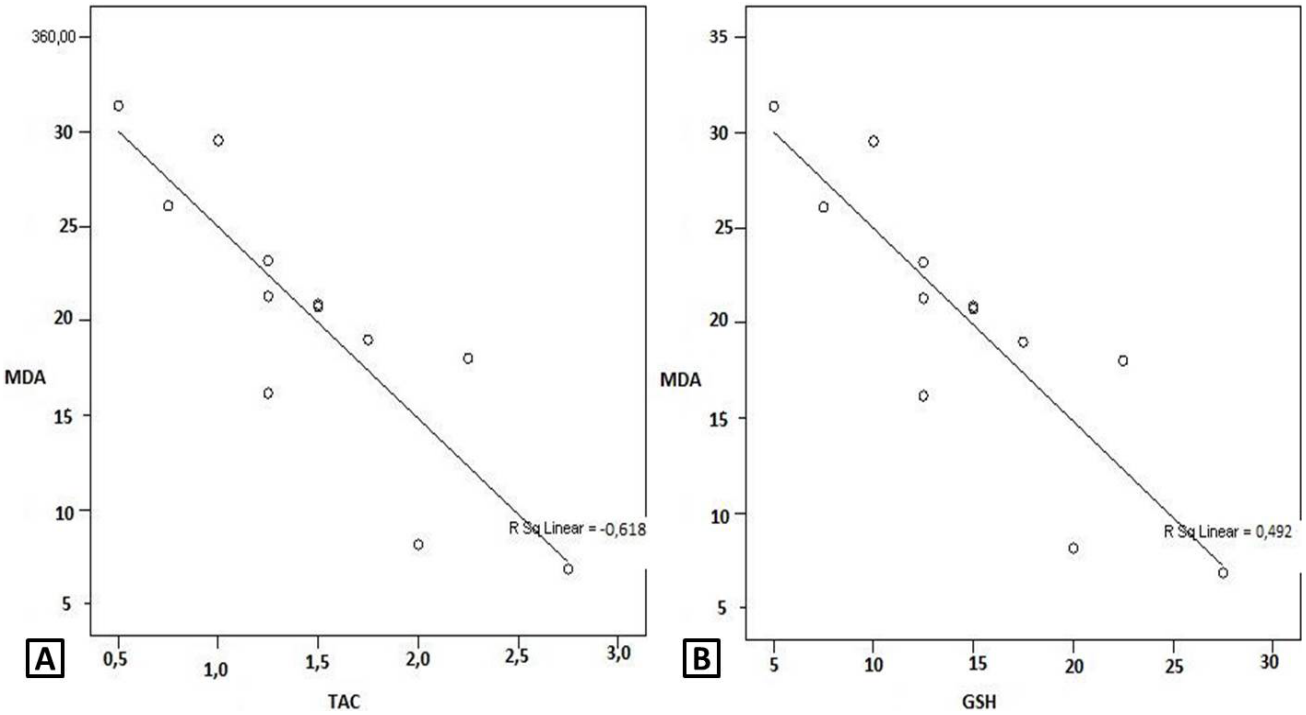

Fig. 2. **A,** the correlation of MDA and TAC level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = -0.618, P<0.05; **B,** the correlation of MDA and GSH level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = -0.492, P<0.001.

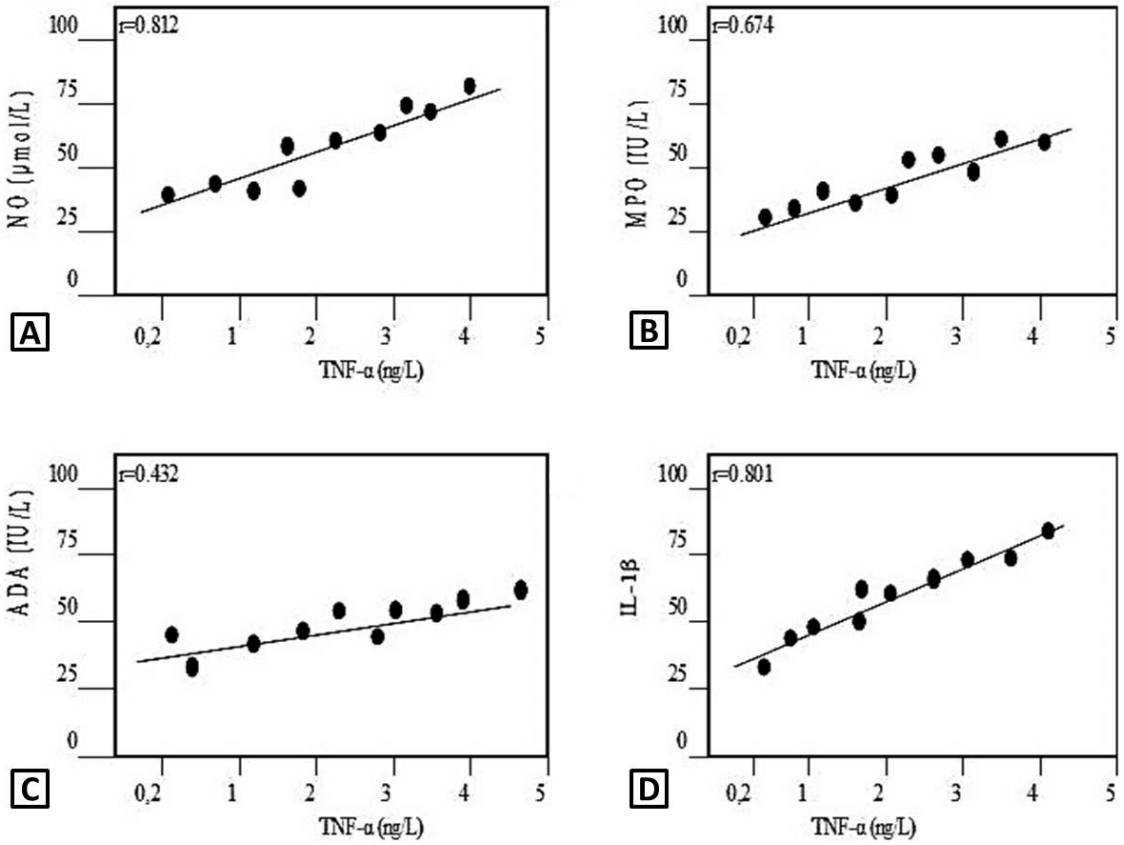

Fig. 3. **A,** the correlation of TNF-α and NO level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = 0.812, P<0.001; **B,** the correlation of TNF-α and MPO level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = 0.674, P<0.01; **C,** the correlation of TNF-α and ADA level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = 0.432, P<0.05.d); **D,** the correlation of TNF-α and IL-1β level in dogs with visceral leishmaniasis. Pearson's rank correlation coefficient was r = 0.801, P<0.001.

However, we analyzed the correlations between the TNF-α and NO, MPO, and ADA concentrations. Pearson's rank correlation coefficient for TNF-α and NO (r=0.812, P<0.001), MPO (r=0.674, P<0.01), ADA (r=0.432, P<0.05), and IL-1β (r=0.801, P<0.001) concentrations, indicating a strong positive correlation was observed. The positive correlation was seen between the TNF-α and NO, MPO, ADA, and IL-1β concentrations as shown in Figure 3.

DISCUSSION

Haematological and serum biochemical measurements in dogs with VL have limited applications for diagnosis, but can be important in understanding the VL pathogenesis (Reis *et al*., 2010). This study was designed to evaluate the oxidative and inflammatory changes in CVL.

Oxidative stress is defined as the inability of the organ or the cell to defend against ROS and to cause oxidative injury (Ozcan *et al*., 2004). Organisms have developed various antioxidant defenses, including non-enzymatic and specific antioxidant enzymes, to provide protection against oxidative damage (Zelko *et al*., 2002). One of the most commonly used ROS biomarkers that is indicative of general lipid peroxidation levels is MDA, a product of lipid peroxidation (Nielsen *et al*., 1997). There are many reports about the increased levels of MDA in cutaneous leishmaniasis (Serarslan *et al*., 2005). In this study, increased levels of MDA and decreased levels of TAC and GSH in CVL (Fig. 1) indicated the presence of oxidative stress and lipid peroxides (Bildik *et al*., 2004; Heidarpour *et al*., 2012); and decreases in TAC and GSH levels during CVL may be related with the prevention of the synthesis of antioxidant enzymes and glutathione by oxidant-induced DNA damage in the chronic stage of the disease (Garça *et al*., 2013).

NO is an important cytotoxic and cytostatic mediator for various intracellular parasites, and the role of NO in Leishmania infection has been evaluated. NO has been

shown to be the main effector molecule responsible for mediating the intracellular killing of Leishmania parasites (Sarkar *et al*., 2011). Studies have shown that there is an interaction between NO and MPO (Brunet, 2001; Knaapen *et al*., 2005). MPO is abundantly present within the cytoplasmatic granules of neutrophils. To the best of our knowledge, no previous report about the serum MPO activity in CVL has been published. In this study, we observed that serum MPO activity was influenced by oxidative stress and NO. Therefore, increased MPO activity may play an important role with NO in the pathogenesis of CVL, and may be related to parasite stimulation of macrophages (Heidarpour *et al*., 2012).

Cytokines play an important role in the development and the regulation of immune response. IL-1β and TNF-α, important inflammatory cytokines, are usually increased in inflammatory diseases of dogs. According to the previous studies, cellular immune response in CVL is associated with activation of Th1 cells producing IFN-γ, IL-1β and TNF-α. IFN-γ, IL-1β and TNF-α are key factors for the initiation, maintenance and persistence of inflammation in leishmaniasis. And these cytokines activate macrophages to generate toxic molecules and ROS that destroy Leishmania parasites inside the macrophages (Erel *et al*., 1999; Reis *et al*., 2010; De Melo and Fortelaza, 2013). ADA enzyme levels are high in many diseases where cellular immunity is stimulated. ADA has been considered as a marker of cell-mediated immunity (Kontaş and Salmanoğlu, 2006) and high levels of ADA activities were well documented in human leishmaniasis (Raziuddin *et al*., 1994). Therefore, we found significantly increase in the levels of serum ADA, IL-1β and TNF-α as MPO and NO levels in dogs with VL in our study (Table I). And this may be related with the phagocytic activity of macrophages and the increased cellular immunity (Erel *et al*., 1999).

CONCLUSION

In the present study, we demonstrated the status of oxidative stress and inflammatory changes in CVL. Decreases in the antioxidant defence and the over production of MDA showed the presence of oxidative stress in CVL. And we consider that free radical causing oxidative stress play an active role in the pathogenesis and immun disorders of CVL. This study is the first report for MPO activity in CVL. Therefore investigation of this enzyme activity in dogs with leishmaniasis may be used for the diagnosis of CVL and inflammatory changes in dogs with VL. Also the increase of activities of MPO and ADA, besides proinflammatory cytokines and NO, can be attributed to respovation of the immune response and parasitemia control. But there is need for further studies on these enzyme activities after different treatment models in CVL.

Statament of conflict of interest

Authors have declared no conflict of interest.

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