

Investigation of DNA damage and protein damage caused by oxidative stress in canine visceral leishmaniasis

FUNDA KIRAL¹, SELIM SEKKIN², SERDAR PASA³, HATICE ERTABAKLAR⁴,
PINAR ALKIM ULUTAS¹, GAMZE SEVRI EKREN ASICI¹

¹Department of Biochemistry, Faculty of Veterinary, University of Aydin Adnan Menderes, Aydin, Turkey

²Department of Pharmacology and Toxicology, Faculty of Veterinary, University of Aydin Adnan Menderes, Aydin, Turkey

³Department of Internal Medicine, Faculty of Veterinary, University of Aydin Adnan Menderes, Aydin, Turkey

⁴Department of Medicine Parasitology, Faculty of Medicine, University of Aydin Adnan Menderes, Aydin, Turkey

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Kiral F., Sekkin S., Pasa S., Ertabaklar H., Ulutas P. A., Ekren Asici G. S.

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Summary

Leishmaniasis, considered by the World Health Organization as one of the most important zoonotic diseases, causes death when it is not treated in its self-healing skin form. The aim of this study was to investigate DNA damage and oxidative protein damage that occurs in dogs infected with *Leishmania infantum*. The study group consisted of 25 dogs, including 10 clinically healthy dogs aged 2 to 6 years and 15 dogs infected with *Leishmania infantum* diagnosed by means of the rk39 dipstick test and the immunofluorescence antibody test (IFAT). The effects of oxidative stress on protein were evaluated in the dogs infected with *Leishmania infantum* by determining plasma malondialdehyde, protein carbonyl groups, nitrotyrosine, and total antioxidant capacity in blood. DNA damage, on the other hand, was determined by the COMET method. Plasma protein carbonyl content (PCO) and nitrotyrosine (NT) levels, which are considered as indicators of protein damage, were found to be higher in the dogs infected with *Leishmania infantum* compared to the control group, but the difference in both values was not statistically significant ($p > 0.05$). Plasma malondialdehyde (MDA), an indicator of lipid peroxidation, was significantly higher in the dogs infected with *Leishmania infantum* than in the control group. TAC levels, however, were lower in the leishmanial dogs ($p < 0.05$). According to the results of the COMET assay, lymphocyte cells were damaged in the leishmanial dogs, and both tail intensity (TI) and tail moment (TM) values were higher in those dogs than they were in the control group ($p < 0.05$, $p < 0.01$). The parasites caused oxidative stress through protein and DNA damage in the host and decreased the antioxidant capacity concentration that prevents destructive effects.

Keywords: canine leishmaniasis, oxidative stress, antioxidant capacity, COMET

Leishmaniasis is regarded by the World Health Organization (WHO) as one of the most important zoonotic diseases. It is an infectious disease affecting particularly dogs, humans, and rodents in most regions of the world, including the Mediterranean region, Central South America, Africa, and Asia. Leishmaniasis is a protozoal disease that frequently has a fatal course. It shows variations ranging from a skin form (cutaneous leishmaniasis, Aleppo boil), which can heal spontaneously, to a form known as visceral leishmaniasis (VL), which affects visceral organs and can cause death if not treated. It is claimed that the early diagnosis of the disease in dogs displaying complex clinical characteristics

plays an important role in the control of leishmaniasis, as dogs are reservoirs of the visceral form (15, 22, 60). The agent of canine visceral leishmaniasis (CanVL) is transmitted from gnats (*Phlebotomus*), the hosts, to dogs during blood-sucking. In dogs, a local skin inflammation develops before the agent spreads to the whole body and signs become visible. The course of the disease may be asymptomatic or symptomatic, depending on the immune system response of animals infected with *Leishmania infantum*. The appearance period of significant clinical signs varies from 1 month to 7 years, depending on the immune status of the host (21, 71, 74).

The diagnosis of VL is complicated because its clinical signs are shared with many other common diseases, such as malaria, typhoid, and tuberculosis (67). Although the accurate diagnosis and characterization of the parasite through direct examination is cheap and easy, this method is not sensitive enough since the number of parasites in the tissue is low (10, 51). Leishmania can be detected in leukocytes in the buffy layer of peripheral blood. If the parasite is not found in the blood, then the spleen, bone marrow, and liver are examined using material taken by lymph node puncture (28, 67). Serological tests, such as Enzyme-Linked Immunosorbent Assay (ELISA), Immunofluorescent Assay (IFA), Direct Fluorescent Antibody Technique (DFAT), and Indirect Fluorescent Antibody Technique (IFAT), are highly sensitive and specific. In these methods, the goal is to facilitate diagnosis by determining antiparasitic antibody titers, which increase in the acute phase. The Polymerase Chain Reaction (PCR) is also widely used in the diagnosis and typing of Leishmania parasites and in the development of specific antigens for serological tests, as well as in examining the cellular immune responses of patients (13, 44, 49).

When cellular antioxidants do not remove free radicals produced during normal metabolic activity, oxidative stress occurs in a cellular system. Since free radicals attack biomolecules (lipids, proteins, nucleic acids) and reduce their bioavailability, oxidative damage occurs in both the protein backbone and the amino acid side chains, and usually at multiple points. The damage which occurs in proteins depends on the nature of the attacking species (18, 73). The most common molecular mechanisms that lead to structural changes in proteins are metal-catalyzed protein oxidation, which is characterized by protein carbonyl content (PCO) formation, the loss of protein thiol (P-SH) groups, the formation of nitrotyrosine (NT), and advanced oxidation protein products (AOPP) (35). Primer modification reactions which lead to the formation of PCO derivatives occur due to the oxidative modifications of α -carbon atoms or side chains of amino acids and due to the reactive oxygen-mediated peptide separation reaction, which occurs after these modifications. Moreover, the formation of carbon-centered radical leads to the formation of protein-protein crosslinks by interacting with another carbon-centered radical (19).

As a result of interaction between reactive oxygen species and proteins, various PCO products are formed depending on oxidative damage occurring in a number of amino acids, such as histidine, proline, arginine, and lysine, and/or in the peptide backbones of proteins (18). NT formation is another molecular mechanism that leads to protein oxidation. ONOO⁻ is a cytotoxic derivative resulting from the *in vivo* reaction of NO[•] and O₂^{-•}. The formation of peroxynitrite causes both the emergence and the development of oxidative protein damage. The basis of the attack on proteins by ONOO⁻ is the nitration of tyrosine in its *ortho* position, which leads

to NT formation. NT concentration is a useful indicator in determining the NO⁻ dependent *in vivo* damage owing to the reality that NT is a stable end product of ONOO⁻ oxidation (38).

Free radicals also attack polyunsaturated fatty acids of membrane lipids that cause lipid peroxidation. Lipid peroxidation impairs the structure and function of the cell, so it is used as a marker of oxidative stress in cells and tissues (4). MDA is an end product of lipid peroxidation if the MDA level in the plasma is directly proportional to lipid peroxidation (55).

All organic molecules, especially DNA, may be susceptible to oxidative damage from a wide variety of oxygen-based (or nitroxygen-based) reactive species under physiological conditions for the duration of life although they have stable structures (4, 12). Many factors that might cause oxidative damage in DNA have been determined. Ionized radiation, high oxygen concentration and structures undergoing auto-oxidation are some of them. They may damage DNA directly by causing excessive free radical formation and they can cause damage by affecting the activity of repair enzymes as well (45). Primary lesions that may occur in DNA as a result of oxidative damage are branch fragments. Later, structural changes occur, such as base pair mutations, rearrangements, deletions, base addition, and sequence amplification. Oxidative DNA modifications can have an antigenic character and cause the formation of anti-DNA antibodies (4). It has been found that oxidative DNA damage has significant effects on the etiology of many diseases, such as rheumatoid arthritis, systemic lupus erythematosus, aging and cancer (45).

In its basic applications related to DNA damage and repair, the comet analysis, along with genotoxicity testing, bio-monitoring, molecular epidemiology and ecogenotoxicity, has recently become one of standard methods of assessing DNA damage. The simplicity, sensitivity, and speed of the method make it increasingly routine (17). The most important advantages of the method are that it does not involve any hazardous operations, such as radioactive tracking, and that it can be applied to easily visible cells. Lymphocytes, which are the most frequently used type of cells, can be easily isolated and are highly compatible with this method (26).

Many studies, including CanVL (6, 7, 10, 11, 33, 62, 63), claim that reactive oxygen species (ROS) play a role in the pathogenesis of various infectious and parasitic diseases in dogs (14, 23, 37, 40, 59, 70). Excessive ROS production leads to oxidative stress as a result of imbalance between oxidants and antioxidants. These reactive species oxidize biomolecules and can thus cause loss of the biological function or various modifications of biomolecules, and even homeostatic imbalance due to structural tissue damage. This study was aimed at investigating DNA and protein damage due to oxidative stress in dogs naturally infected with *Leishmania infantum*.

Material and methods

Blood samples were taken from 15 dogs that had been naturally infected with *Leishmania infantum* and developed visceral leishmaniasis (VL). They were 2-6 years old, except for 2, which were 8 years old. Ten dogs were used as a healthy control group. Seven of them were 3-4 years old, and 3 were 6 years old. All the animals were selected from an area in western Turkey where VL is endemic. Physical examination of the infected dogs was performed at the beginning of the study. Dogs that had been ill for approximately four months were not given any treatment when they were included in the study. The clinical diagnosis was confirmed serologically by the immunofluorescence test (IFAT) for antibodies to *Leishmania infantum* as described in detail elsewhere (Antibody titer = 64 was considered as a positive test) (1). In the control group, all animals showed negative responses, and in the infected group, all dogs tested positive. Blood samples from the dogs were taken into heparinized tubes, and a certain amount of blood was reserved for leukocyte isolation using Histopaque 1077 and the comet method within 2 hours, while the rest was centrifuged at 1500 g for 10 minutes to obtain plasma samples. The study protocol, including procedures for animal handling and husbandry, was reviewed and approved by the Animal Care and Use Committee of Adnan Menderes University (approval number 050.04/2010/006).

Measurement of plasma protein carbonyl content (PCO). Protein carbonyl proteins were assayed by spectrophotometric detection. Protein CO groups react with 2,4-dinitrophenylhydrazine (DNPH), and then a stable dinitrophenyl (DNP) hydrazone product is formed (43). The carbonyl content was expressed in nmol mg⁻¹ protein. The total protein content was measured with a colorimetric kit by the biuret method.

Measurement of plasma nitrotyrosine. The NT content in plasma was determined by the competitive immunoassay method in comparison with a standard curve. The standard curve was determined with values of nitrated bovine serum albumin taken from a kit at determined concentration (Oxi-Select™ Nitrotyrosine ELISA Kit, STA-305; Cell Biolabs, Inc., San Diego, CA, USA).

Measurement of plasma malondialdehyde (MDA). Lipid peroxidation in plasma samples was determined according to the method of Ohkawa et al. (55). The basic principle of this method is the spectrophotometric determination of colored compounds that can be measured at 532 nm, which are formed as a result of the reaction of TBA and the final product of lipid peroxidation. Absorbance was detected with a spectrophotometer (Shimadzu UV-1601). In this assay, tetraethoxypropane was used as a standard.

Measurement of total antioxidant capacity (TAC) of plasma. The total antioxidant capacity of plasma was measured by a novel automated colorimetric measurement method for TAC developed by Erel (24). In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown. Upon the addition of the plasma sample, oxidative reactions, initiated by hydroxyl radicals present in the reaction, are suppressed by the antioxidant components of the plasma, preventing color change and

thus providing an effective measurement of TAC. The assay results were expressed in mmol Trolox Eq/l.

Lymphocyte isolation and DNA analysis by comet assay. The level of DNA damage in lymphocyte cells was determined by the comet method used by Singh et al. (69). It is an accepted method of measuring DNA damage and repair in a single eukaryotic cell and in some prokaryotic cells. A small number of cells suspended in a thin layer of agar gel on a microscope slide were subjected to electrophoresis after lysis, and the broken and lightened DNA fragments were rapidly removed from the core. For this purpose, fresh blood samples were mixed v/v (1/1) with phosphate buffered saline solution to determine the DNA fragmentation of blood lymphocytes. Lymphocytes were isolated with histopaque and suspended in a freezing medium (PBS with 10% foetal calf serum and 15% DMSO). All the above processes were completed within 2 hours after the blood sample was taken into a heparinized tube. After isolation, the cells were counted in thoma lam under a light microscope right after lymphocyte solution and cells were re-suspended in PBS at a concentration of 1 × 10⁶ cells/ml. Before use, all slides were dipped in ethanol and dried in burner flame in order to remove possible impurities on the slides. In order to embed the lymphocyte cells and to ensure migration during electrophoresis, the top faces of the slides were covered with 1% normal melting agarose, which dissolves in a 60°C water bath. Slides that were ready were stored at 4°C. Once the gel had solidified at 4°C, the cover slip was removed, and the slides were dipped in freshly prepared lysis solution jars at 4°C for at least 1 h. Positive control slide cells were dipped in H₂O₂ (50 and 100 mM in PBS) solution for 5 min at 4°C, then washed with cold PBS and immersed in a lysis solution in a separate jar for at least 1 h at 4°C. After lysis, the slides were aligned in a horizontal gel electrophoresis tank connected to a recirculating cooler set at 4°C and filled with freshly made alkaline electrophoresis solution (pH > 13). Electrophoresis was carried out at approximately 1 V/cm for 20 min, after which the slides were washed twice with 0.4 M Tris HCl buffer (pH 7.5) and then fixed with different concentrations (50%, 75% and 90%) of ethanol. They were then allowed to dry at room temperature prior to staining with 70 ml of ethidium bromide solution (20 mg/ml). To visualize DNA damage, the slides were examined at a 400-fold magnification under a fluorescence microscope (DM3000, Leica, Germany). The tail intensity and tail moment of comets were measured for 100 randomly selected cells, 50 cells from each of the two gels from each sample, with a computer-based 2 M. image analysis system (Comet Assay IV, Perceptive Instruments, UK).

Statistical analyses. The findings of the study were analyzed using the SPSS 21 program (Standart Version Copyright© SPSS Inc. Chicago, IL, USA). The differences between the two study groups were determined by means of Student's t-test, with p < 0.05 as the limit of significance.

Results and discussion

All animals with visceral leishmaniasis showed some clinical manifestations of the disease, such as skin lesions, weight loss, lymphadenopathy, and anaemia. Leishmaniasis in dogs can be diagnosed by a number

of different methods: cytohistological methods, serological tests, parasitological methods (xenodiagnosis, culture), molecular methods, and determination of cellular immune response (2, 64). The sensitivity and specificity of the IFAT method are reported to be close to 100%, and it is considered to be the reference serological method by the World Animal Health Organization (WOAH) (47). Titers of 1 : 64 and above, which are agreed to be adequate for the diagnosis of leishmaniasis in dogs as a result of comparisons of clinical findings (29, 31, 36) with laboratory tests, were also accepted as positive in this study.

Plasma MDA, TAK, PCO, NT levels and t-test results for the dogs included in the study are shown in Table 1. The mean concentration of MDA in the leishmanial dogs was significantly higher ($p < 0.05$) than in the controls. The dogs with CanVL had significantly lower levels of TAC. As seen in Table 1, the sick dogs had significantly higher levels of PCO and NT ($p < 0.05$). DNA damage levels in lymphocyte samples from the healthy and leishmanial dogs were determined by the comet method. DNA damage was significantly greater in the infected animals than in the controls ($p < 0.05$). The findings for the dogs included in the study are shown in Table 2.

Parasites cause damage in the organs, tissues, and cells of the host, and the oxidative stress due to this damage leads to lipid peroxidation (20, 48). Oxygen radicals are important mediators of host defense and tissue damage. Lipid peroxide products are used as an indicator of oxygen radical activation in clinical tables of various animal diseases. Malondialdehyde is one of the most important products of lipid peroxidation. The TAC value represents the capacity of antioxidant molecules in the sample to reduce an oxidant. It also makes it possible to determine the levels of antioxidants that have not yet been identified or are difficult to analyze. In addition, it represents the general antioxidant status of plasma and body fluids, as it provides information about the synergistic interactions of these antioxidants (32). Similar studies on oxidative stress in dogs with canine visceral leishmaniasis reported that lipid peroxidation increased (9, 12, 50), whereas albumin and total antioxidant capacity decreased (3, 34, 58, 72). In this study, as well, the MDA level, which is an indicator of lipid peroxidation, was found to be significantly higher in the dogs with leishmaniasis compared to the control group, while TAC values were lower in the sick dogs.

Nowadays, comprehensive studies are carried out on metal-catalyzed protein oxidation, which is characterized by PCO production and leads to changes in the protein structure. As a result of the attack of free radicals on proteins, some amino acid residues in the chain, such as histidine, proline, arginine, and lysine, are damaged and, consequently, amino acid residues and/or oxidative degradation of the peptide chain in proteins leads to the formation of PCO products. Therefore, the

Tab. 1. Comparison of plasma and blood parameters in dogs with visceral leishmaniasis (VL) and healthy controls

	Controls	CanVL Dogs	p
Malondialdehyde ($\mu\text{mol/L}$)	25.28 \pm 2.46	41.74 \pm 4.45	$p < 0.05$
Total antioxidant capacity (mmol trolox equivalent/L)	0.55 \pm 0.10	0.40 \pm 0.09	$p < 0.05$
Protein carbonyl group (nmol/mg)	26.74 \pm 1.18	29.04 \pm 0.53	$p > 0.05$
Nitrotyrosine (nM)	3.75 \pm 0.26	4.05 \pm 0.26	$p > 0.05$

Tab. 2. Tail intensity and tail moments of lymphocytes isolated from the control and leishmanial dogs (mean \pm SE)

Group	Parameter	
	% Tail intensity	Tail moment
Control (n = 10)	19.73 \pm 1.24 ^b	4.19 \pm 0.53 ^b
VL (n = 15)	36.97 \pm 4.89 ^a	12.54 \pm 2.62 ^a
p	*	**

Explanations: ^{a, b} values with different letters show statistically significant differences; * – $p < 0.05$, ** – $p < 0.01$

PCO concentration can accurately indicate the level of oxidative degradation in proteins (39).

In a study on dogs infected with *Ehrlichia canis*, Silva et al. (68) showed that, in parasitic diseases, protein damage increased along with increase in advanced oxidation protein products (AOPP). However, thus far, there have been no reports on protein oxidation in canine visceral leishmaniasis. Only a single study on patients with cutaneous leishmaniasis by Kocyigit et al. (42) is known. In the present study, the PCO content in plasma was higher in the leishmanial dogs compared to the control group, but the difference was not statistically significant. Our findings are in line with those by Kocyigit et al. (42).

NT formation, another molecular mechanism causing oxidative protein damage, is another indicator on which studies have been conducted in recent years. Peroxynitrite formation with the combination of superoxide radical and nitric oxide has various effects on both the onset and the progression of oxidative protein damage. The nitration of the tyrosine ortho position is the most important production channel of peroxynitrite, which attacks proteins and leads to NT formation. Even though the *in vivo* determination of peroxynitrite may be useful in assessing the level of protein damage, its ability to easily oxidize the thiol groups of the peroxynitrite structure, lipids, proteins, and DNA means that it is highly toxic. The determination of the NT concentration *in vivo* is regarded as more practical in assessing the level of NO-dependent damage, since NT is a stable end product of peroxynitrite oxidation (39). No study on NT levels in dogs infected with *Leishmania* has been found in the literature, although there are studies in which various clinical tables and serum NT levels are examined in tooth diseases in dogs (52), gastrointestinal disorders (66), acute lung injury (53), and heart diseases (56). In this study, plasma NT values in dogs with leishmaniasis were higher, but statistically insignificant. This increase

in NT levels can be linked to a decrease in antioxidant capacity and the nitric acid level that increases with the stimulation of nitric oxide synthase enzyme.

As a result of the attacks by free radicals on the DNA molecule, which has a vulnerable structure, mutations and cell deaths take place. Furthermore, chromosomal changes, fractures in the DNA chain, errors in repair, replication, and transcription of DNA are observed, together with the interaction of DNA with glycosylation products in DM. Oxidative DNA damage is a useful indicator of oxidative stress and the risk of cancer. It is also observed that the oxidation of DNA bases increases in patients with diabetes and Alzheimer (27).

The comet analysis, which is used in measuring the level of oxidative DNA damage, is sensitive and fast, and its error rate is low. In addition, this method is a valuable tool in population monitoring, such as assessing the role of oxidative stress in human diseases and examining antioxidant effects (16). In this study, oxidative DNA damage was determined by the comet analysis and the size of the damage was indicated by tail density and moment values. The results of the analysis show that the integrity of lymphocyte cells in the leishmanial dogs was disrupted, and both tail intensity and moment values were higher than they were in the control group. Although there is no report on DNA damage and canine visceral leishmaniasis infection in the literature, it is reported that some other infectious diseases lead to DNA damage. Farinati et al. (25) reported that DNA damage can be induced by free radicals produced by polymorphonuclear cells of *Helicobacter pylori*. In a study by Pinloar et al. (61), it was demonstrated, by stimulating nitric oxide synthase expression in hamsters infected with *Opisthorchis viverrini*, that nitrative and oxidative DNA damage is related to cholangiocarcinoma. Riberio et al. (65) studied the genotoxic effects of *Try. cruzi* in mice that they had experimentally infected with this parasite and found that tail moment values in the peripheral blood, heart, and spleen tissues of infected mice were elevated. Our results indicate that ROS and RNS were produced excessively as a result of the long-term activation of inflammatory cells due to the host defense in patients with canine visceral leishmaniasis. However, these reactive species not only kill the parasite, but also increase DNA damage in other cells and tissues. This study is the first to evaluate endogenous DNA breaks in patients with canine visceral leishmaniasis.

In conclusion, this study supports the view that ROS and RNS produced by the organism as a defense strategy can increase leishmanicidal activity in patients with canine visceral leishmaniasis. However, these intermediate oxides could cause not only the death of parasites, but also DNA damage in adjacent cells. Oxidative stress developing in these patients and the associated DNA and protein damage are important factors in understanding the pathology and pathogenesis of CanVL and can contribute to possible treatment strategies.

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Corresponding author: Gamze Sevri Ekren Aşıcı PhD, Research Assistant, Aydın Adnan Menderes University, Veterinary Medicine Faculty, Biochemistry Department, Aydın, Turkey; e-mail: fkargin@adu.edu.tr