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Original Article

The Effects of Babesiosis on Oxidative Stress and DNA Damage in Anatolian Black Goats Naturally Infected with *Babesia ovis*

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Abstract

Background: A reactive oxygen and nitrogen intermediate produced during an inflammatory response is the important part of host-defense strategies of organisms to kill the parasite. However, it is not well known whether these intermediates cause DNA damage and oxidative stress in goats infected with *Babesia ovis*. The purpose of this study was to clarify the effects of babesiosis on basal levels of DNA damage and oxidative status of goats naturally infected with *B.ovis*.

***Methods*:** DNA damage and antioxidant parameters were determined in *B. ovis* infected goats. Ten infected *Anatolian Black Goats* with *B. ovis* diagnosed via clinical signs and microscopic findings and ten healthy were used in the study.

***Results*:** The *Babesia* infection increased the levels of DNA damage, malondialdehyde (MDA), protein carbonyl content (PCO) and plasma concentration of nitric oxide metabolites (NOx), and decreased total antioxidant activities (AOA) and reduced glutathione (GSH). A significant positive correlation between DNA damage, MDA, PCO, and NOx concentrations was found in the infected goats. DNA damage showed a negative association with AOA and GSH concentrations in the infected goats.

***Conclusion*:** The *Babesia* infection increases oxidative stress markers and DNA damage and decreases AOA in goats. These results suggest that the increases in the production of free radicals due to *Babesia* infection not only contribute to host-defense strategies of organisms to kill the parasite but also induce oxidative damage in other cells.

Introduction

Babesiosis is caused by a haemotropic protozoal parasite of the genus *Babesia*, member of the phylum Apicomplexa and transmitted by the bite of an infected tick" (1). There are many *Babesia* spp. affecting animals which are of economic significance. Babesiosis can occur without producing symptoms, but also be severe and sometimes fatal due to the intraerythrocytic parasite development (1). The disease can cause fever, inappetence, increased respiratory rate, muscle tremors, anemia, jaundice, body weight loss and hemolytic anemia lasting from several days to several months (1, 2). All babesial parasites described to date are transmitted by ixodid ticks to their vertebrate hosts, and replicate in the vertebrate host's red blood cells (3). In babesiosis, there is an increase in the erythrocyte membrane permeability and phagocytosis of the erythrocytes by activated macrophages. In conclusion, the erythrocytes are destroyed by the physical effect of parasite multiplication (4).

A possible role of the reactive oxygen and nitrogen species (ROS and RNS) in the pathogenesis of parasitic infections has been an active area of research in recent years (5, 6). In addition, the mechanisms by which cellular defense kills microorganisms have been the subject of intense research. Numerous studies have demonstrated that a variety of inflammatory cells are induced or activated by various oxidant-generating enzymes to kill intra-cellular and extra-cellular parasites (7). The reactive species are produced primarily to attack invading microorganisms by nitration, oxidation and chlorination reactions. However, excess amounts of ROS and RNS can cause an injury to host cells and lead to tissue damage (7-9). In fact, there are some studies determined the effects of babesiosis on oxidative markers and antioxidants (10, 11), but there is no report available on DNA damage in goats infected with *Babesia* spp. Therefore, in this study, we investigated the role of ROS and RNS generating by macrophages as a first line of defense during phagocytosis in parasitic infections on DNA strand breaks, total antioxidant activity (AOA), reduced glutathione (GSH), protein carbonyl (PCO), nitric oxide (NOx) and malondialdehyde (MDA) in *Babesia* infected goats.

Materials and Methods

Chemicals

Vanadium (III) chloride (VCl_3), sodium chloride (NaCl), Ethylenediaminetetraacetic acid disodium (EDTA-Na_2), sodium hydroxide (NaOH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrochloric acide (HCl), sulfanilamide (SULF), and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) were obtained from Merck (Darmstadt, Germany). Dinitrophenlyhydrazine (DNPH) was purchased from Fluka (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Other chemicals used in the study were purchased from Sigma-Aldrich Chemical.

Subjects and parasitological examination

The study was conducted in Afyonkarahisar, Turkey, July and September 2009. Ten *Anatolian Black goats* naturally infected with *B. ovis*, at the age of 2-3 years were used in the study. *Babesia* infection was diagnosed in the goats via clinical and microscopic findings. Altogether ten healthy *Anatolian Black* goats aged 2-3 years from the same area served as controls. They were not exposed to *Babesia* infection and none had treatment with either mineral or vitamin drugs. The study protocol was performed in accordance with the Helsinki declaration as revised in 1989.

On clinical examination, the infected goats showed signs of babesiosis including rise in rectal temperature, dyspnea, tachycardia, anorexia, muscle tremors, signs of anaemia in pale mucous membranes and brownish urine. The control goats were healthy on clinical exami-

nation. *Babesia* infection was detected by examination of Giemsa-stained blood films. Blood smears of all the selected animals were prepared aseptically on grease free sterile glass slides directly from the ear vein. The blood smears of infected goats were taken at the phase of haemoglobinuria. Each slide was labeled with specific number and date of smear made. The smears were air dried, fixed in methanol and stained with freshly prepared 5% Giemsa stain for 45 minutes. After being stained with Giemsa, the smears were examined for *B. ovis* under a research microscope and 100X objective. The parasites were identified according to the characters described by Levine (12).

Sample collection and biochemical estimation

Blood samples were taken from each animal by puncture of the jugular vein into heparinized tubes. Two milliliters of blood was immediately pipetted into another tube to measure MDA, GSH and DNA damage. The remaining blood was centrifuged at 3000 rpm for 10 min for plasma separation. Plasma samples were stored at -30°C for the analysis of PCO, AOA and NOx.

DNA damage determination by alkaline comet assay

The endogenous lymphocyte DNA damage was analyzed by alkaline comet assay using a similar method described by Singh et al. (13) with the following modifications. Samples were processed within 2 h and lymphocyte isolation for the comet assay was performed by using the Histopaque 1077. An amount of 1 ml blood was carefully layered over 1 ml histopaque and centrifuged for 35 min at 500×g and 25 °C. The interface band containing lymphocyte was washed with phosphate buffered saline (PBS) and then collected by 15-min centrifugation at 400×g. The resulting pellets were resuspended in PBS to obtain 20000 cells in 10 µl. Membrane integrity was assessed trypan blue exclusion method and revealed membrane integrity in 95% of cells. Ten microliters of fresh lymphocyte cell suspension was mixed with 80 µl of 0.7% low-melting-point agarose in PBS at 37 °C. Subsequently, 80 µl of this mixture was layered onto slides that had previously been coated with 1.0% hot (60 °C) normal-melting-point agarose, and then covered with a coverslip at 4 °C for at least 5 min to allow the agarose to solidify. After removing the coverslips, the slides were submerged in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA-Na_2; 10 mM Tris-HCl, pH 10-10.5; 1% Triton X-100 and 10% DMSO were added just before use for at least 1 h). The slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH and 1 mmol/l ETDA-Na_2, pH 13) at 4 °C for 30 min and then electrophoresed (25 V/300 mA, 25 min). All the steps were carried out under minimal illumination. After electrophoresis, the slides were then neutralized (0.4 M/L Tris-HCI, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 µg/ml solved in distilled H_2O; 70 µl/slide), covered with a coverslip, and analyzed using a fluorescence microscope (Olympus, Japan). The images of 100 nuclei chosen randomly were analyzed visually. Each image was classified according to the intensity of the fluorescence in the damaged nuclei and was given a value of 0, 1, 2, 3, or 4 so that the total scores of the slide could be between 0 and 400 arbitrary units (AU) (7).

Determination of malondialdehyde levels

MDA levels, an index of lipid peroxidation, were measured by the double heating method of Draper and Hadley (14). The method is based on spectrophotometric measurement of the purple color generated by the reaction of TBA with MDA. For this purpose, 2.5 ml of TCA solution (10%, w/v) was added to 0.5 ml whole blood in each centrifuge tube. The tubes were then placed in a boiling water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at 1000×g for 10

min and 2 ml of each sample supernatant was transferred to a test tube containing 1 ml of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm by using the Shimadzu UV 1601 spectrophotometer. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex (ε=1.56×105 cm/M).

Measurement of protein carbonyl content

Protein carbonyls were measured by using the method of Levin et al. (15). Briefly, 15 µl of plasma was placed in each of the two glass tubes. Then 0.5 ml of 10 mM DNPH in 2.5 M HCl was added to one of the tubes, while 0.5 ml HCl (2.5 mM) was added to the second tube. Tubes were incubated for 1 h at room temperature. Samples were vortexed every 15 min. Then 0.5 ml TCA (20%, w/v) was added and the tubes were left on ice for 5 min followed by centrifugation for 5 min to collect the protein precipitates. The pellet was then washed three times with 2 ml ethanol–ethyl acetate (1:1, v/v). The final precipitate was dissolved in 1 ml of guanidine hydrochloride solution (6 M) and was incubated for 15 min at 37°C while mixing. The absorbance of the sample was measured at 365 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH (ε=2.2×104 cm/M).

Determination of plasma total antioxidant activity

The total AOA was determined using the method described by Koracevic et al. (16). The assay measures the capacity of the serum to inhibit the production of TBA reactive substances (TBARS) from sodium benzoate under the influence of the oxygen-free radicals derived from Fenton's reaction. The reaction was measured spectrophotometrically at 532 nm. The antioxidants from the added sample suppress the production of TBARS, and the inhibition of color development is defined as AOA. A solution of 1 mmol/l uric acid was used as standard.

Determination of blood-reduced glutathione levels

GSH concentration of erythrocyte was measured using the method described by Beutler et al. (17). Briefly, 0.2 ml of whole blood was added to 1.8 ml distilled water. Precipitating solution of 3 ml (1.67 g metaphosphoric acid, 0.2 g EDTA, and 30 g NaCl in 100 ml distilled water) was mixed with haemolysate.

The mixture was allowed to stand for approximately 5 min, filtered (Whatman No. 42), and 2 ml of the filtrate was transferred into another tube, after which 8 ml of the phosphate solution (0.3 M disodium hydrogen phosphate) and 1 ml DTNB were added. A blank was prepared with 8 ml of phosphate solution, 2 ml of diluted precipitating solution (three parts to two parts distilled water), and 1 ml of DTNB reagent. A standard solution of the GSH was prepared (40 mg/100 ml). The optical density was measured at 412 nm in the spectrophotometer

Estimation of plasma nitric oxide levels

Nitric oxide decomposes rapidly in aerated solutions to form stable nitrite/nitrate products (NOx). Plasma nitrite/nitrate concentration was measured by a modified method of Griess assay, described by Miranda et al. (18). The principle of this assay is the reduction of nitrate by vanadium combined with detection by the acidic Griess reaction. Briefly, samples were deproteinized before the assay. The serum was added to 96% cold ethanol at 1:2 (v/v) and then vortexed for 5 min. After incubating for 30 min at 4°C, the mixture was centrifuged at 8000×g for 5 min and the supernatants were used for the Griess assay. Analysis was done in a microtiter plate. One hundred microliters of filtrated plasma was mixed with 100 µl of VCl_3 and was rapidly followed by the addition of the Griess reagents, which

contain SULF 50 µl and NEDD 50 µl. The determination was performed at 37°C for 30 min. The absorbance was measured by a microplate reader (Multiskan Spectrum, Thermo Labsystems, Finland) at 540 nm. Nitrite/ nitrate concentration was calculated using a $NaNO_2$ standard curve.

Statistical analysis

All data were presented separately as mean ± SE for the infected and the healthy groups. The comparisons of parameters were performed with Student's t-test and the correlation analyses by Pearson's correlation test. Data were analyzed using the SPSS for Windows computing program (Version 10.0) and $P<0.05$ was considered statistically significant (19).

Results

The results of whole blood MDA, DNA damage, GSH, PCO, serum AOA and plasma NOx levels in controls and infected groups are summarized in Table 1.

As seen in Table 1, the amount of endogenous mononuclear leukocyte DNA damage and levels of MDA, PCO and plasma NOx concentrations were higher in the infected goats than in the healthy goats ($P<0.05$). Furthermore, AOA and GSH concentrations were lower in the infected goats as compared to the healthy goats.

The correlation coefficients obtained between biochemical parameters in the infected goats are presented in Table 2.

Table 1: The effects of *B. ovis* on whole blood DNA damage, MDA, PCO, GSH, serum AOA and plasma NOx concentrations in healthy and infected goats (n:10)

Parameters	Infected group	Healthy control group
DNA damage (AU)	95.32±7.65*	21.37±3.54
MDA (nmol/ml)	9.59±0.63*	5.06±0.24
PCO (nmol/mg protein)	1.38±0.05*	1.10±0.01
GSH (g/dl)	19.53±1.73*	30.7±2.13
AOA (mmol/L)	1.56±0.13*	3.10±0.14
NOx (µmol/L)	47.06±3.11*	36.44±2.04

Values are shown as ±SE. (*: $P < 0.05$). DNA: deoxyribonucleic acid; MDA: malondialdehyde; PCO: protein carbonyl; GSH: reduced glutathione; AOA: total antioxidant activity; NOx: nitrite/nitrate products

Table 2: Correlation coefficients obtained between biochemical parameters in the infected goats (n: 10)

	MDA	GSH	AOA	PCO	NOx	DNA Damage
MDA	1	-0.763 $P<0.05$	-0.792 $P<0.05$	0.759 $P<0.05$	0.886 $P<0.05$	0.766 $P<0.05$
GSH		1	0.796 $P<0.05$	-0.696 NS	-0.801 $P<0.05$	-0.769 $P<0.05$
AOA			1	-0.823 $P<0.05$	-0.618 NS	-0.797 $P<0.05$
PCO				1	0.723 $P<0.05$	0.803 $P<0.05$
NOx					1	0.735 $P<0.05$
DNA Damage						1

DNA: deoxyribonucleic acid; MDA: malondialdehyde; PCO: protein carbonyl; GSH: reduced glutathione; AOA: total antioxidant activity; NOx: nitrite/nitrate products

MDA levels were positively correlated with DNA strand breaks, PCO and NOx in the *Babesia* group ($r = 0.766$, $P < 0.05$, $r = 0.759$, $P < 0.05$ and $r = 0.886$, $P < 0.05$, respectively). Besides these data, MDA levels were negatively associated with concentrations of AOA and GSH ($r = -0.792$, $P < 0.05$ and $r = -0.763$, $P < 0.05$, respectively). GSH levels in the infected group were positively correlated with AOA ($r = 0.796$, $P < 0.05$).

However, GSH levels were negatively correlated with DNA strand breaks and NOx ($r = -0.769$, $P < 0.05$ and $r = -0.801$, $P < 0.01$, respectively). AOA was negatively correlated with PCO and DNA strand breaks in the infected group ($r = -0.823$, $P < 0.05$, $r = 0.797$, $P < 0.05$ respectively). The correlations of NOx and DNA strand breaks in the infected group with PCO were positive ($r = 0.723$, $P < 0.05$ and $r = 0.803$, $P < 0.05$, respectively). There was significantly positive correlation between NOx and DNA strand breaks in the infected group ($r = 0.735$, $P < 0.05$).

Discussion

Parasitic infections cause an activation of inflammatory cells which play an important role in the host defense (4, 9). Activation of inflammatory cells induces and activates various oxidant-generating enzymes. The induction and activation of these enzymes in inflammatory cells are also regulated by many pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and others (7). In babesiosis caused by *B. bovis,* the infection involves production of IL-1β, IL-12, IFN-γ and TNF-α (20, 21). The enhanced production of such cytokines might increase cancer risk by inducing or activating enzymes involved in the production of inflammatory cytokines because these enzymes produce large amounts of highly toxic molecules, such as ROS including superoxide anion, hydrogen peroxide and hydroxyl radicals, and RNS, including nitric oxide (7, 20, 21, 22). Excess ROS and RNS productions are capable of degrading numerous biomolecules, including DNA, carbohydrates, lipids and proteins (23, 24). Koçyiğit et al. (7) demonstrated that *Cutaneous leishmaniasis* infection could induce DNA damage through the production of free radicals generated by polymorphonuclear cells. Kim et al. (25) studied the mutagenicity of ROS and RNS produced by stimulated leukocytes and observed that stimulated human promyelocytic leukemia cells and LPS/IFN-g-stimulated murine macrophages induced 8-OHdG formation in transgenic Chinese hamster ovary cells mediated by ROS and RNS. The results in the present study showed that DNA damage increased significantly in infected goats with *B. ovis*. This result is the first report on the assessment of DNA strand breaks in *Babesia* infection.

In the cells of hosts infected with different species of parasites, the amount of reactive oxygen species (ROS), thereby causing cell and tissue damage (9, 26). ROS induce the oxidation of polyunsaturated fatty acids in biological systems and lead to the formation of lipid peroxidation (LP) products. One of the most frequently used ROS biomarkers, providing an indication of the overall LP levels, is MDA, one of several from products of LP (27). In the present study, *Babesia* infection increased blood MDA levels. This result is an agreement with the result in cattle infected with *B. bigemina* (4) and indicates that LP increases in *Babesia* infection. Ames et al. (28) reported that LP is closely related to the formation of cancer and degradation products of hydroxyl aldehydes, which indirectly damage DNA and stimulate cancer gene expression. In the present study, there was also a positive correlation between MDA levels, PCO content and DNA damage in infected goats. Considering the close relationship between DNA damage and MDA, it could be stated that an elevated level of DNA damage in goats infected with *B. ovis* might depend on the oxidative radicals generated during the course of the disease.

Free radicals react with proteins and modify amino acid residues by oxidation, nitrosation, and carbonylation. The oxidative inactivation of enzymes and the oxidative modification of proteins cause the formation of protein carbonyl derivatives (15). In the present study, *Babesia* infection increased the plasma PCO content in the goats. This result suggests that *Babesia* infection causes the alterations in structure and function of proteins induced by free radicals.

Nitric oxide (NO) is an important mediator of both physiological and pathophysiological processes. Macrophages, neutrophils and mast cells have all been shown to be major producers of this molecule (29). The NO levels increased a tissue dysfunction or injury in the organism (30). In the present study, *Babesia* infection increased the plasma NOx concentration in the goats. This result is an agreement with the results of studies reported that nitrate concentration, one of NO metabolites, increases in blood of animals infected with parasites (31).

The antioxidant defense system includes small molecular antioxidants, antioxidant enzymes, and metal chelating agents. The total AOA of body fluids suggests a simultaneous interaction between various antioxidants and is crucial for the maximum suppression of a free radical reaction in extracellular compartments. Such an activity appears to indicate the antioxidant characteristics of only one antioxidant, whereas total antioxidant activity represents the aggregate antioxidant characteristics of all antioxidants found in the plasma. In addition to AOA and reduced GSH exhibit the major defense against ROS-induced cellular damage. GSH is a nonenzymatic antioxidant and serves as a reductant in oxidation reactions resulting in the formation of GSSG. Therefore, GSH can protect cells against the damage from ROS and free radicals that arise during conditions of oxidative stress (32, 33), and thus reduced GSH levels may reflect a depletion of the antioxidant reserve. As a consequence of GSH deficiency, a number of related functions may be impaired protein biosynthesis, immune function, accumulations of lipid peroxidation products and detoxification capacity (34, 35). Das et al., (36) expressed that parasites damage the cells which synthesis the molecules carrying the anti-oxidative agents. In the present study, *Babesia* infection decreased the AOA levels and GSH concentrations. This result is agreement with the results reported that the GSH concentration fell in animals infected with parasite species (9, 37-39) and suggests that *Babesia* infection causes a significant depletion of the antioxidant reserve of the host.

Conclusion

Babesia infection increased DNA damage, MDA, PCO and concentration of NOx from the oxidative stress markers and decreased AOA and GSH concentrations from the antioxidant markers in the present study. These results indicate that overproduction of reactive oxygen and nitrogen species by activated neutrophils and macrophages due to *Babesia* infection increases oxidative stress, and thus leads to the acceleration of lipid peroxidation, DNA damage and protein oxidation in the host. The positive relationship between DNA damage and oxidants as well as the negative relationship with antioxidants supports our hypothesis, which claims that the oxidants generated in babesiosis could cause a significant damage in DNA.

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