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RESEARCH ARTICLE

The Correlation betweenNeopterin, Myeloperoxidase and Oxidative DNA Damage in Sheep with Natural Babesiosis

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Doğal Babesiozisli Koyunlarda Neopterin, Myeloperoksidaz ve Oksidatif DNA Hasarı Arasındaki ;İlişki

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Öz

Amaç: Bu çalışma doğal babesiozisli koyunlarda Neopterin, MPO ve DNA hasarı arasında bir ilişki olup olmadığının belirlenmesi amacıyla planlandı.

Gereç ve Yöntem: Çalışmaya 1-3 yaşlarında Akkaraman 20 sağlıklı koyun (Kontrol) ve 20 doğal babesiozisli (Hasta) olmak üzere 40 koyun dahil edildi. Tüm çalışma gruplarından *V. Jugularis*'ten usulüne uygun biyokimya tüplerine kan örnekler alındı. Yapılan froti sonucuna göre etken belirlenen hayvanlarda ve ayrıca hastalık etkeni PCR sonucuna göre doğrulandı. Sağlıklı ve hastalıklı hayvanlardan elde edilen serumlardan Neopterin, MPO ve oksidatif DNA hasarı (8-OHdG) ELISA methodu kullanılarak belirlendi.

Bulgular: Neopterin ve 8-OHdG seviyeleri, kontrol grubunda hasta grubuna kıyasla anlamlı derecede daha düşük tespit edildi (P<0.05). Myeloperoksidaz seviyesi, hasta grubunda daha yüksek olmasına rağmen, bu fark istatistiksel olarak anlamlı değildi (P>0.05)

Öneri: Bu çalışmada, babesiosisli koyunlarda immünolojik yanıtın göstergeleri olan neopterin ve oksidatif DNA hasarında önemli bir artış gözlemlendi. Özellikle neopterin, babesiosis'e karşı immünolojik yanıtın önemli bir belirteci olarak düşünülebilir.

Anahtar kelimeler: Babesiosis, DNA hasarı, koyun, miyeloperoksidaz, neopterin

Abstract

Aim: The present study was designed to investigate the correlation between neopterin, myeloperoxidase (MPO), and DNA damage in sheep naturally infected with babesiosis.

Materials and Methods: The study included 40 sheep Akkaraman, 1-3 years aged, with 20 healthy individuals (control group) and 20 naturally infected with babesiosis (patient group). Blood samples were collected from the jugular vein into biochemical tubes from all sheep. The cause of the disease was confirmed through smear tests and PCR analysis. Neopterin, myeloperoxidase (MPO), and oxidative DNA damage (8-OHdG) levels were measured using an ELISA method.

Results: Neopterin and 8-OHdG levels were significantly lower in the control group compared to the patient group (P<0.05). Although myeloperoxidase were higher in the patient group, the difference was not statistically significant (P>0.05)

Conclusion: In this study, a significant increase in neopterin and oxidative DNA damage, which are indicators of an immunological response, was observed in sheep with babesiosis. Neopterin in particular can be considered as an important marker of the immunological response to babesiosis.

Keywords: Babesiosis, DNA damage, myeloperoxidase, neopterin, sheep

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Introduction

Babesia species are protozoan parasites that infect red blood cells and are transmitted by ixodid ticks through both transstadial and transovarian routes. The most commonly encountered species worldwide are *Babesia ovis* and *Babesia motasi* (Çiçek et al 2004; Kılınç et al 2018). These parasites can cause significant mortality in sheep and goats in tropical and subtropical regions, leading to severe economic losses. In Turkey, babesiosis is observed as a seasonal disease, particularly affecting sheep across all regions (Sevinc and Xuan 2015). The hallmark symptoms of babesiosis include anemia, high fever, and hemoglobinuria. If left untreated, affected animals may develop shock and ultimately die from renal failure (Yur et al 2010; Esmaeilnejad et al 2012).

MPO enzyme is a lysosomal enzyme secreted from leukocytes in response to oxidative stress. MPO, which is abundantly found in leukocytes and forms reactive oxidant products, is involved in atherosclerotic reactions and exhibits catalytic activity (Kargapolova et al 2021). MPO levels rise continuously due to the release of active neutrophils from azurophil granules during inflammation and infection (Nauseef 1998). The most notable property of MPO is its ability to catalyze the oxidation of various substrates and halogens in conjunction with hydrogen peroxide (Davies 2011; Eiserich et al 2002). In addition to generating reactive oxygen species, MPO is involved in the formation of neutrophil extracellular traps (NETs) (Metzler et al 2011). NETs are extracellular structures composed of chromatin and antimicrobial proteins released by active neutrophils, and they play a role in binding and neutralizing pathogens (Brinkmann et al 2004).

Neopterin, a member of the pteridine chemical group, is a marker of immunological processes involving monocytes, macrophages, and dendritic cells. It is synthesized by macrophages in response to interferon-gamma (IFN-γ) produced by activated T cells, and it is associated with immune activation (Weiss et al 1999). Recent research has shown that neopterin plays a significant role in oxidative stress. In macrophages, neopterin enhances the cytotoxicity of reactive oxygen species during the respiratory burst, thereby amplifying the cytotoxic effects of cell-mediated immune responses. Various studies across different fields have investigated neopterin as a marker of both cell-mediated immunity and oxidative stress (Sucher 2010).

DNA repair is a cellular defense mechanism responding to DNA damage caused in large part by oxidative stress. (Gafter-Gvili et al 2013). One of the most extensively studied metabolites of oxidative DNA damage is 8-hydroxy-2-deoxyguanosine (8-OHdG), as guanosine is the most susceptible to oxidation among the DNA nucleobases (Kasai 1997). The present study aimed to investigate whether there is a correlation between neopterin, myeloperoxidase, and DNA damage in sheep affected by natural babesiosis.

Material and Methods

In this study, twenty Konya Akkaraman sheep aged between one and three years, exhibiting typical symptoms of babesiosis such as fever, anemia, hemoglobinuria, and jaundice, were selected. Blood smears were prepared from the ear tips of the sheep and fixed in methanol, then stained with Giemsa to confirm the presence of Babesia parasites. Morphological and biometric parameters, including the shape and location of the parasite in infected erythrocytes, were considered for differential diagnosis. Additionally, PCR analysis was conducted to further confirm the diagnosis. No species identification was performed in this study. As a control group, twenty clinically healthy Akkaraman sheep from tick-free farms were used. For PCR analysis, 5 ml of blood was collected in EDTA tubes, and for biochemical analyses, 10 ml of blood was drawn from the jugular vein into biochemical tubes. Serum was obtained by centrifugation at 3000 × g for 10 min. Serum samples were preserved at –20 °C until analysis.

DNA extraction from blood samples was performed using the GeneJet Viral DNA and RNA Purification Kit (Thermo Scientific, Cat. no: K0821) following the manufacturer's instructions. PCR amplification of nucleic acids was conducted with primers BJ1 (5'-GTCTTGTAATTGGAATGATGG-3') and BN2 (5'-TAGTTTATGGTTAGGACTACG-3'), which are specific to the 18S rRNA gene region (Schorn et al 2011). A total of 50 µl PCR reaction mix was prepared, consisting of 5 µl DNA, 75 mM Tris-HCl (pH 8.8), 20 mM ammonium sulfate, 1.5 mM MgCl2, 10 pmol of each primer, 0.2 mM dNTPs, and 0.5 U Taq DNA polymerase (MBI, Fermentas, Lithuania). The PCR reaction was denatured in a thermocycler with an initial 5 cycles at 94°C. This was followed by 30 cycles consisting of 30 seconds at 94°C, 30 seconds at 55°C, and 40 seconds at 72°C. This cycle was repeated for a total of 40 cycles. After the final extension at 72°C for 10 minutes, a DNA product of 425 base pairs was obtained. For evaluation, a 100 bp DNA ladder was used as a reference. The amplified DNA products were stained with ethidium bromide, separated on a 1% agarose gel, and visualized under UV light using a gel imager.

Neopterin, Myeloperoxidase, and DNA damage (8-OHdG) were analyzed using the ELISA method with BioTek Elx-800 equipment, following the manufacturer's recommended procedures (Rel Assay Diagnostik).

The 8-OHdG kit (Cat No: E0151Sh) in sheep serum demonstrates high sensitivity and specificity for measuring 8-OHdG levels. The sensitivity is 0.94 ng/mL, and the detection range is 1.56-100 ng/mL. The Neopterin ELISA kit (Cat No: LS-F56490) offers high sensitivity and specificity for

measuring neopterin levels in sheep serum. The sensitivity is 0.1 nmol/L, and the detection range is 0.5-80 nmol/L. The Myeloperoxidase ELISA kit (Cat No: E0005Sh)provides high sensitivity and specificity for measuring myeloperoxidase levels in sheep serum. The sensitivity is 1.0 ng/mL, and the detection range is 6.25 ng/mL to 200 ng/mL.

Statistical Analysis

In this study, descriptive statistics such as mean and standard deviation were calculated for data obtained from both the control group and the group with babesiosis. The normality of the data was assessed using the Shapiro-Wilk test. The differences between the groups were analyzed using Student's t-test. A statistical significance level of 5% $p<0.05$ was established for the analyses. The computations wereperformed using the SPSS (version 20) statistical software.

Results

Serum levels of both neopterin and 8-OHdG were significantly higher in sheep with babesiosis compared to the control group ($P<0.05$) (Table 1 and Figure 1 and 2)

Serum MPO levels were elevated in sheep with babesiosis compared to the control group, but the difference was not statistically significant ($P>0.05$) (Table 1 and Figure 3).

Discussion

It is widely accepted that the NADPH oxidase system and hydrogen peroxide (H_2O_2) generated by myeloperoxidase (MPO) are crucial for microbial mortality (Pacheco-Yepez et al 2014). MPO, a cationic enzyme found in the azurophilic granules of neutrophils and immature monocytes, plays a significant role in immune responses (Campos-Rodriguez et al 2016). Upon release by neutrophils, MPO interacts with monocytes that express reactive oxygen species and mannose receptors, potentially leading to the release of proinflammatory cytokines (Kumar and Sharma 2010). Elevated levels of MPO may enhance the destruction of parasites (Theeß et al 2016) and are integral to natural immunity and pathogen defense (Klebanoff et al 2013).

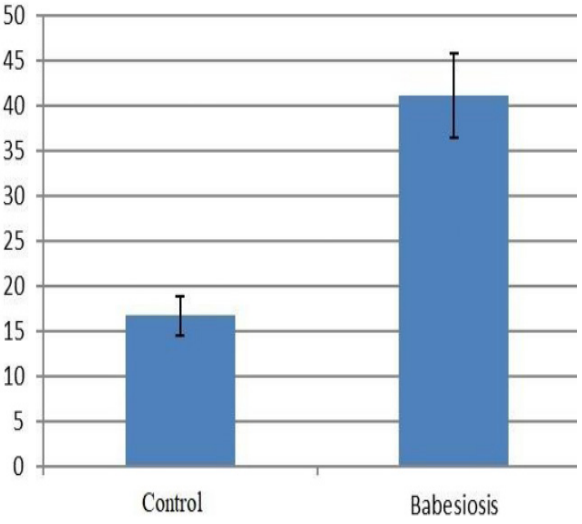

Figure 1. Serum neopterin level in health and natural babesiosis sheep

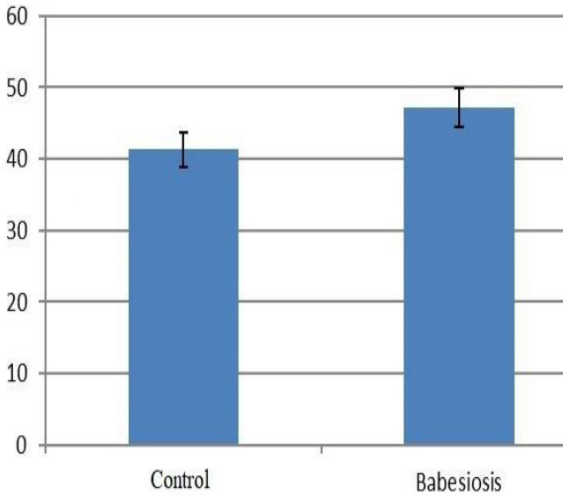

Figure 2. Serum 8-OHdG level in health and natural babesiosis sheep

Although MPO itself has low bactericidal activity, it generates hypohalitic acids such as hypochlorous acid (HOCl), hypobromous acid (HBr), and hypoiodous acid (HOI) through enzymatic reactions with hydrogen peroxide (H_2O_2), halides (Cl, Br, I), and pseudohalides (SCN) (Kettle and Winterbourn, 1997). These hypohalitic acids are thought to be primarily responsible for the antibacterial effects of neutrophils (Davies 2011).

Table 1.Serum Neopterin, MPO and 8-OHdG values in health and natural babesiosis sheep.

Parameters	Control	Babesiosis	P
Neopterin (nmol/L)	16.71±2.20	41.15±4.67	<0.05
MPO (ng/ml)	104.62±7.82	105.23±7.27	>0.05
8-OHdG (ng/ml)	41.29±2.40	47.16±2.63	<0.05

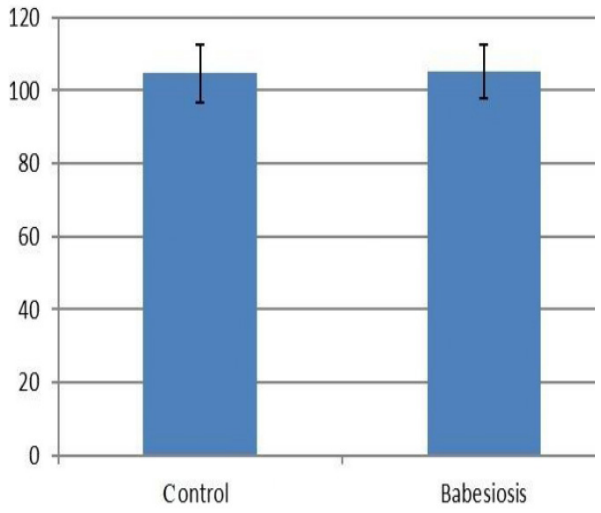

Figure 3. Serum MPO level in health and natural babesiosis sheep

Myeloperoxidase (MPO) plays a crucial role in the oxidative destruction of pathogens by neutrophils (Klebanoff et al 2013) and may contribute to the elimination of parasites. Studies have shown that MPO can inhibit parasite survival (Locksley et al 1987) and that MPO plasma levels are elevated in patients with *Plasmodium falciparum* infections. Increased MPO production has been associated with the accelerated removal of *Plasmodium* parasites (Theeß et al 2016). In the present study, serum MPO levels were found to be higher in sheep infected with *Babesia ovis* compared to the control group, although the difference was not statistically significant (Figure 3). In cases of low pathogen exposure, it has been suggested that MPO may not be essential for preventing infections. The crucial role of MPO in natural host defense becomes more apparent when pathogen levels surpass the host's defense mechanisms (Klebanoff et al 2013). The lack of a significant difference in MPO levels in the babesiosis group in the present study might indicate that the infection was either at a low level or not severe enough to overwhelm the host's other defense mechanisms.

Neopterin has been reported to activate the immune response in various pathological conditions, including HIV infection (Berdowska and Zwirska-Korczala 2001; Hagberg et al 2010). Elevated urine neopterin levels have been observed in patients infected with *Plasmodium falciparum* and *P. vivax* compared to controls (Reibnegger et al 1984). Neopterin is synthesized and released by dendritic cells and macrophage-derived monocytes in response to stimulation by interferon-gamma (IFN-γ), a cytokine produced by T cells (Aleksandrova et al 2015). It is associated with viral infections and the activation of cellular immunity, including intracellular bacterial and parasitic infections. High concentrations of neopterin are linked to reduced antioxidant levels and increased production of reactive oxygen species, making it a recognized biomarker of oxidative stress that activates the cellular immune system (Murr et al 1999). Neopterin may also serve as an indicator of infections caused by external pathogens (Ip et al 2007). In the present study, serum neopterin levels were significantly higher in *B. ovis*-infected sheep compared to the control group (Figure 1). The increase in neopterin is indicative of the activation of cell-mediated immunity. The elevated neopterin levels in *B. ovis* infected sheep are attributed to the infection's role in increasing oxidative stress.

Reactive oxygen radicals are crucial in the pathogenesis of parasitic infections due to their role in oxidative DNA damage. The production of reactive oxygen species (ROS) increases in various pathological conditions, leading to lipid peroxidation and the formation of oxidative DNA damage products (Halliwell and Gutteridge 1985). 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a metabolite resulting from oxidative DNA damage induced by free radicals (Valavanidis et al 2009).

Parasite infections trigger the activation of inflammatory cells, which play a crucial role in host defense. This activation leads to the upregulation of various oxidant-producing enzymes. These enzymes generate highly toxic molecules, including RNS such as hydroxyl radicals containing nitric oxide, and ROS such as hydrogen peroxide and superoxide anions (Küçükkurt et al 2014). Additionally, Kocyigit et al (2005) demonstrated that cutaneous leishmaniasis infection can induce DNA damage in polymorphonuclear cells. Kim et al (2003) demonstrated that ROS and RNS produced by activated leukocytes can induce DNA damage. Another study found that DNA damage increased in sheep infected with *B. ovis* (Küçükkurt et al 2014). The production of ROS in host cells infected with various parasitic species leads to cellular and tissue damage (Bildik et al 2004; Değer et al 2001). Elevated DNA damage levels in *B. ovis* infected sheep have been attributed to oxidative radicals generated during the disease (Biçek et al 2005). Additionally, it has been reported that 8-hydroxyguanine (8-OHdG), a critical biomarker of oxidative stress that reacts with DNA nitrogen bases, increases in babesiosis and that Babesia spp. causes DNA damage (Esmaeilnejad et al 2020; Küçükkurt et al 2014). In a study examining DNA damage in animals diagnosed with babesiosis, tail length and tail moment values were found to be statistically significantly higher compared to the control group (Öner et al 2022).

Several studies have reported increased DNA damage in infections caused by *Plasmodium* and *Trypanosoma* (Yano et al 2008; Herbas et al 2009; Herbas et al 2010), as well as in dogs infected with *B. vogeli* (Ciftci etl al 2014) and sheep naturally infected with *B. ovis* (Küçükkurt et al 2014). These studies attribute the increased DNA damage to oxidative radicals produced during the course of the disease. In the

present study, the level of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was significantly higher in B. ovis-infected sheep, which is consistent with the findings of the aforementioned studies (Table 1).

Conclusion

In this study, a significant increase in neopterin and oxidative DNA damage, which are indicators of an immunological response, was observed in sheep with babesiosis. Neopterin in particular can be considered as an important marker of the immunological response to babesiosis.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Author Contributions

Motivation/Concept: SC, AUK; Design: SC, VY; Control/Supervision: SD, NY, OOK; Data Collection and Processing: SC, VY, AUK; Analysis and Interpretation: SC, SD, AUK; Literature Review: AUK, SC; Writing the Article: AUK, SC; Critical Review: SD, NY, OOK

Ethical Approval

Ethics committee approval for this study was obtained from Van Yuzuncu Yil University, Animal Experiments Local Ethics Committee (Van YUHADYEK) on 01/02/2024 (Decision No:2024/01-10)