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RESEARCH ARTICLE

Effect of Imidocarb on DNA Damage in Sheep with Babesiosis

Ahmet Cihat ÖNER [1,a (*)] Adnan AYAN [2,b] Özlem ORUNÇ KILINÇ [3,c]
Ayşe USTA [4,d] Fatma ERTAŞ [5,e]

[1] Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, TR-65080 Van - TÜRKİYE
[2] Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Department of Genetics, TR-65080 Van - TÜRKİYE
[3] Van Yüzüncü Yıl University Özalp Vocational School of Higher Education, Department of Medical Laboratory Technician, TR-65800 Van - TÜRKİYE
[4] Van Yüzüncü Yıl University, Faculty of Science, Department of Chemistry, TR-65080 Van - TÜRKİYE
[5] Igdır University, Tuzluca Vocational School of Higher Education, TR-76000 Iğdır - TÜRKİYE

ORCIDs: [a] 0000-0001-6614-4347; [b] 0000-0002-6564-3416; [c] 0000-0001-6233-7109; [d] 0000-0002-5522-3469; [e] 0000-0001-5289071X

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Abstract

In this study, it was aimed to determine the DNA damage using the comet assay, which specifically shows DNA damage in naturally *Babesia* spp.-infected sheep and to evaluate the damage before and after imidocarb application. Blood samples obtained from 10 infected sheep with positive clinical signs and symptoms of babesiosis and whose diagnosis was confirmed by Giemsa staining and PCR methods, and blood samples from 10 healthy sheep were used as study material. DNA damage was examined by the comet assay from the blood samples of the infected patient group and the control group obtained during the disease and after the treatment, and the results were compared with statistical methods. When DNA damage was examined in sick animals diagnosed with babesiosis, the tail length and the tail moment values were found to be statistically significantly higher than the control group ($P<0.001$). According to the results obtained after imidocarb application, it was determined that DNA damage and tail moment decreased statistically with imidocarb, and the difference was statistically significant, and the values were higher than the control group ($P<0.001$). As a result, *Babesia* infection can cause DNA damage, has been confirmed by the determination of direct DNA damage using the comet assay, and imidocarb given for treatment was successful and reduced the damage.

Keywords: *Babesiosis, DNA damage, Imidocarb, Sheep*

Babeziozisli Koyunlarda İmidokarb Uygulamasının DNA Hasarına Etkisi

Öz

Bu çalışmada, doğal olarak *Babesia* spp. ile enfekte koyunlarda spesifik olarak DNA hasarını gösteren comet testi kullanılarak DNA hasarının belirlenmesi ve imidokarb uygulaması öncesi ve sonrası hasarın değerlendirilmesi amaçlanmıştır. Çalışma materyali olarak babeziozis klinik belirti ve semptomları pozitif olan ve Giemsa boyama ve PCR yöntemleri ile tanısı doğrulanan 10 enfekte koyundan alınan kan örnekleri ve 10 sağlıklı koyundan alınan kan örnekleri kullanıldı. Enfekte hasta grubu ve kontrol grubundan hastalık sırasında ve tedavi sonrasında alınan kan örneklerinden comet testi ile DNA hasarı incelendi ve sonuçlar istatistiksel yöntemlerle karşılaştırıldı. Babeziozis tanısı konulan hasta hayvanlarda DNA hasarı incelendiğinde kuyruk uzunluğu ve kuyruk momenti değerleri kontrol grubuna göre istatistiksel olarak anlamlı derecede yüksek bulundu ($P<0.001$). İmidokarb uygulaması sonrası elde edilen sonuçlara göre DNA hasarı ve kuyruk momentinin imidokarb ile istatistiksel olarak azaldığı ve aradaki farkın istatistiksel olarak anlamlı olduğu ve değerlerin kontrol grubuna göre daha yüksek olduğu belirlendi ($P<0.001$). Sonuç olarak *Babesia* enfeksiyonunun DNA hasarına neden olabileceği, comet testi kullanılarak direkt DNA hasarının belirlenmesi ile doğrulanmış ve tedavi için verilen imidocarb başarılı olmuş ve hasarı azaltmıştır.

Anahtar sözcükler: *Babeziozis, DNA hasarı, İmidokarb, Koyun*

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[(*)] **Corresponding Author**

Tel: +90 432 225 1128/21589 Cell Phone: +90 542 535 4042 Fax: +90 432 225 1127
E-mail: ahmetcihatoner@yyu.edu.tr (A. C. Öner)

INTRODUCTION

Babesiosis is a tick-borne haemoparasitic disease that causes high morbidity and mortality and high economic losses in tropical and subtropical regions of the world, and it is the most critical blood-borne parasitic disease of small ruminants [1,2]. Six species of *Babesia (B. ovis, B. motasi, Babesia (Lintan), B. crassa, B. foliata*, and *B. taylori)* have been described, of which *B. ovis* and *B. motasi* have been reported to be pathogenic *Babesia* species [2-4]. Babesiosis is a disease that causes high economic losses in the livestock industry worldwide. Clinically, symptoms such as fever, anemia, jaundice, and hemoglobinuria are observed in babesiosis. Babesiosis can be seen as a "protozoan sepsis" in different animals and is expressed to be clinically similar to septic conditions characterized by systemic inflammatory response syndrome (SIRS) and multiple organ failure syndrome (MODS) [5].

Microscopic examination of samples stained with Giemsa stain is the most commonly used method in the diagnosis of babesiosis, but this method is not specific. Since there are false negative results in low-density parasitemia and some *Babesia* species cannot be distinguished from Theileria, serological methods and molecular-based tests such as PCR have frequently been used in epidemiological studies in recent years [6]. Molecular-based tests are more sensitive and allow identification of species by using appropriate primers [7-9]. Early diagnosis and successful treatment of babesiosis reduces the mortality rates. Clinicians use imidocarb in the treatment of babesiosis. Imidocarb is a carbanilide derivative and is usually available in the form of the dipropionate salt. For the treatment of babesiosis in sheep, intramuscular (IM) administration of 1.2 mg/kg once is recommended. However, a second dose may be given 10 to 14 days later for disease control. It is stated that it can be used at a dose of 2.4 mg/kg for prophylaxis of the disease in sheep [10].

DNA is a sensitive molecule and DNA damage can occur for various reasons. DNA damage leads to necrosis or cellular mutation when damage is high or repair systems are insufficient and this plays an important role in mutagenesis, carcinogenesis and aging [11-13]. Free radicals can attack any macromolecule, including DNA, and can cause lipid peroxidation, protein oxidation and DNA damage [14-17]. DNA damage is characterized by structural damage such as disruption of chromatin structure, oxidation of DNA bases, mismatch and suppression of tubulin polymerization, chemical modification of bases, chromatin abnormalities, strand breakage, DNA-DNA and DNA-protein crossovers [18-20]. Parasitic infections cause activation of inflammatory cells that play an important role in host defense. In addition, parasites increase the amount of free radicals in the tissues, organs and cells they inhabit and cause lipid peroxidation, which causes tissue and cell damage in the host. It has been reported that erythrocyte membrane fragility occurs as a result of increased lipid peroxidation and decreased antioxidant defense in the erythrocytes of animals with piroplasmosis [21-23]. Increased activation of inflammatory cells and therefore, increased oxidant-producing enzymes, have been reported in sheep infected with *Babesia* spp., but the extent of DNA damage has not been determined by specific methods [24].

The Comet assay [single-cell gel electrophoresis (SCGE)], which is among the methods used for detection of DNA damage, is a fast, simple, sensitive and widely used technique. The comet assay method is based on the principle that DNA molecules with different molecular weights and different electrical charges migrate differently in the electrical field at alkaline pH. While healthy DNAs do not form comets during transport, damaged DNAs move at different speeds in the electrical field, forming a tail-shaped image. DNA degradation, antioxidant status (resistance to H_2O_2 degradation) and DNA repair levels in lymphocytes can also be measured with the comet assay [25,26].

Babesiosis is common worldwide, especially among small livestock, and causes serious economic losses. It has been reported that oxidative stress occurs in *Babesia* spp. infections, but studies showing precise DNA damage are insufficient in number [27]. In this study, it was aimed to determine DNA damage in sheep naturally infected with *Babesia* spp. using the comet assay, which specifically shows DNA damage, and to evaluate the damage before and after imidocarb application.

MATERIAL AND METHODS

Sample Collection and Identification of Babesia

In this study, 10 mature Akkaraman sheep with a weight of 25-40 kg, aged 3-5 years, showing clinical babesiosis symptoms (40-42°C fever, anemia, hemoglobinuria, jaundice, etc.), located in a farm in the Özalp District of Van, Türkiye in July 2021 were included as the patient group. The control group consisted of 10 healthy sheep, which were subject to the same region and rearing conditions, had no disease history and clinical findings specific to babesiosis and other diseases, and were found to be negative for *Anaplasma* spp. and *Theileria* spp. with microscopic examination (5% Giemsa stain) and blood samples were obtained from these sheep for analysis. Before and after the treatment (Day 10), blood samples were taken from the sheep diagnosed with the disease for laboratory analysis. All tracked animals were kept in their natural habitat for the duration of the study. The study was performed with the Van YYU Animal Experiments Local Ethics Committee (VAN YUHADYEK) decision (It was decided that ethics committee approval was not required) (Approval no: 2020/12-08, date: 31/12/2020).

Microscopic Diagnosis of Babesia spp.

Blood smear staining was performed with 5% Giemsa stain by taking blood samples from the Vena jugularis of the animals. Piroplasma forms were found in erythrocytes with microscopic examination.

Molecular Diagnosis of Babesia spp.

- DNA Extraction

PCR test was performed by isolating DNA from all samples suspicious for *Babesia* by microscopic examination using the Invitrogen PureLink™ Genomic DNA Mini Kit (USA, K182002), according to the manufacturer's protocol.

- PCR Reaction

Orunç Kılınç et al.[28] performed amplification of the 18S rRNA gene region, using BJ 5'-GTCTTGTAATTGGAATGATGG-3' and BN2 5'-TAGTTTATGGTTAGGACTACG-3' primers [29]. A 5 pmol forward and reverse primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1U Taq Polymerase and 10X PCR buffer (500 mM Tris-HCl, pH 8.8, 160 mM $(NH_4)SO_4$ and 0.1% Tween®20), Nuclease Free Water and 2 µL of DNA were used in 25 µL master mix for one sample. At the end of the microscopic examination were PCR tested with positive animals, negative animals, in addition to 1 positive and 1 negative control. The reaction was followed by pre-denaturation at 95°C for 15 min, with each cycle consisting of denaturation (30 sec at 95°C), bonding (30 sec at 55°C) and elongation (40 sec at 72°C) steps, in 40 cycles and a final extension of 10 min at 72°C. The obtained PCR products were stained with Safe-T-Stain and images were obtained on 2% agarose gel.

- DNA Damage Analysis

The Comet analysis method was used to determine DNA damage. It was applied on gel-coated slides according to the Comet protocol and spread was achieved. Prepared slides were run by the electrophoresis method [30]. Three times the sample volume LMA was added and mixed with Whole Blood with EDTA. It was added to slides that had been applied with NMA. 3 samples were studied from each group. The slides were scanned with a fluorescence microscope, and visual damage levels were counted (Oxion Microscopy for Fluorescence, The Netherlands). DNA damage levels were calculated based on the genetic damage index (GDI) formula. The genetic damage index reflects the number of Arbitrary Units [15]. The % DNA Damage and the % Tail Moment measurements from these images were calculated using the "Image J" program (a program distributed freely by the National Institute of Health of the SA *(https://imagej.nih.gov/ij/download.html)*.

Statistical Analysis

All results are reported as mean ± standard error of the mean. The data of each sampling time of all groups were evaluated with the One-Way Anova test. The significance of the difference between the groups was evaluated with the Duncan test (SPSS® v.19 Evaluation Version for Windows, IBM).

Results

In the present study, clinically high fever >40°C, hemoglobinuria, jaundice, increased heart and respiratory rate were determined in the patient group. The smears obtained from blood samples taken from animals with clinical symptoms were stained with the Giemsa staining method and examined microscopically, and piroplasms were observed in erythrocytes *(Fig.1)*. In order to confirm the results of the microscopic examination, in the PCR test performed on suspicious blood, as a result of the amplification of the 18S rRNA gene region, specific fragments specific for *Babesia* spp. were obtained with a size of approximately 447 bp in all 10 samples *(Fig. 2)*.

DNA damage in sheep with babesiosis after the comet analysis has been demonstrated in *Fig. 3*. When DNA damage *(Table 1)* and tail moment *(Table 2)* were examined

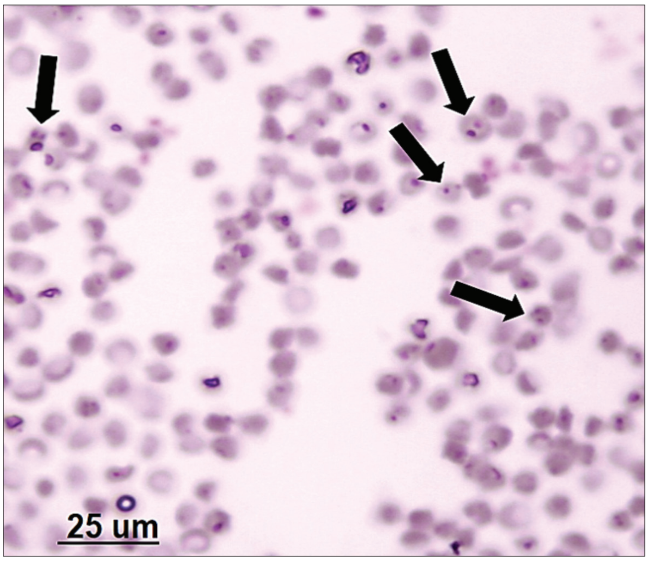

Fig 1. *Babesia* spp. (Giemsa staining)

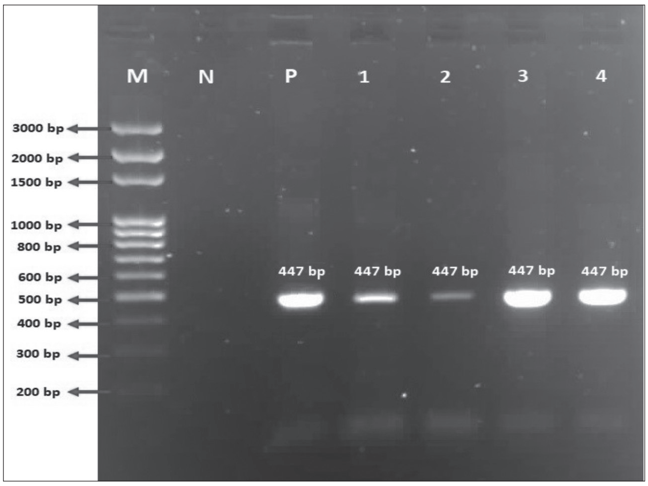

Fig 2. *Babesia* spp. agarose gel image (M: marker, P: positive control, N: negative control, 1,2,3,4: positive samples)

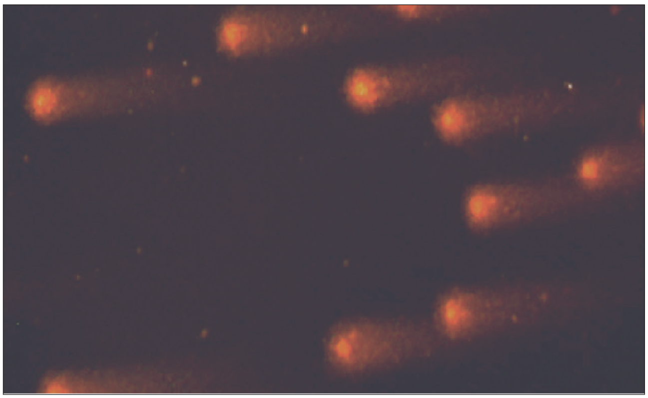

Fig 3. DNA damage in sheep with babesiosis (the comet analysis)

Table 1. *Imidocarb application DNA damage table in babesiosis treatment (n:10)*

Groups	Mean	Std. Error
Control	5.71784	0.7169242 [c]
Patient	36.2785	1.5562375 [a]
Treatment	16.58399	0.8135902 [b]

[a,b,c] Indicates the difference between groups P<0.001

Table 2. *Imidocarb application tail moment table in babesiosis treatment (n:10)*

Groups	Mean	Std. Error
Control	6.68563	0.4814676 [c]
Patient	39.46755	1.4941459 [a]
Treatment	16.48585	0.5343389 [b]

[a,b,c] Indicates the difference between groups P<0.001

in sick animals diagnosed with babesiosis, the values were found to be statistically significantly higher than that of the control group (P<0.001). After imidocarb administration, it was determined that DNA damage, which was found to be significantly different to sick animals as the treatment group, decreased, but there was a significant difference compared to the control group and its value was higher (P<0.001) *(Table 2)*.

Discussion

Free radicals react with proteins and lead to modification of amino acid residues by oxidation, nitrosation and carbonylation. In fact, protein carbonyl (PCO) derivatives are produced when enzymes and proteins are deactivated and modified by free radicals. In addition, oxidative DNA damage can cause a range of changes including mutations, replication errors, genomic instability and cell death [31]. DNA damage may be associated with hydroxyl radicals (•OH) produced in parasitic infections [32]. It has been reported that the level of 8-hydroxyguanine (8-OHG), which can react with DNA nitrogen bases and is one of the critical biomarkers of oxidative stress, increases in babesiosis and the *Babesia* spp. causes DNA damage [33,34].

Küçükkurt et al.[34] found that *Babesia* infection increased the oxidative stress markers and DNA damage and decreased (total antioxidant activity) AOA and glutathione (GSH) in goats, and that the increase in the production of free radicals generated during infection not only contributed to the host defense strategies of organisms to kill the parasite, but also induced leads to the acceleration of lipid peroxidation in other cells. As a result, they reported a DNA damage in goats with comet assay. In our study, DNA damage occurring in sheep with babesiosis was detected by the comet assay *(Fig. 3, Table 1, Table 2)*, and these results support the literature information mentioned above.

Ostling and Johanson [35] were the first to measure DNA damage in cells using a microgel electrophoresis technique known as "single-cell gel electrophoresis" or "Comet assay". However, the neutral conditions they used allowed detection of only DNA double-stranded breaks. Later, this method was adapted under alkaline conditions by Singh et al.[36]. It led to a sensitive version of the analysis that could evaluate both double- and single-stranded DNA breaks, as well as alkaline variable regions in DNA, expressed as open-strand breaks. However, this method has been modified at several stages (lysis, electrophoresis) to make it suitable for assessing various types of damage in different cells [25,26]. There are previous studies reporting that DNA damage may occur in babesiosis, but these studies investigated oxidative stress and 8-OHG markers [34-37]. Comet, on the other hand, is a method that reveals specific DNA damage, and in this study, it has been confirmed that DNA damage occurs in babesiosis *(Table 1, Table 2, Fig. 3)*.

There are many studies on babesiosis and imidocarb administration. In one study, it was shown that imidocarb dipropionate (IMD) was more effective compared to diminazene aceturate [38]. In another study, it was reported that the use of imidocarb dipropionate together with oxytetracycline produced more successful results, and it was more effective than the combined use of diminazene aceturate and oxytetracycline [39]. In another study, the combined application of imidocarb and alpha-lipoic acid (ALA) was reported to be successful in treatment in dogs experimentally infected with *Babesia canis vogeli* [40]. In our study, the presence and extent of DNA damage during infection and after treatment in sheep with babesiosis was investigated, and it was revealed that DNA damage occurred during infection and this damage decreased after treatment; hence, imidocarb application was successful *(Table 1, Table 2)*.

In this study, blood samples were obtained from 10 sick Akkaraman sheep, in which *Babesia* spp. were diagnosed microscopically and molecularly *(Fig. 1, Fig. 2)*, and DNA damage was examined using the comet method, and blood samples were obtained from 10 healthy sheep. As a result of the study, when DNA damage *(Table 1)* and tail moment *(Table 2)* were examined in sick animals diagnosed with babesiosis, the values were found to be statistically significantly higher than the control group (P<0.001). After imidocarb administration, it was determined that DNA damage, which was found to be significantly different in sick animals, decreased, but there was a significant difference compared to the control group and its value was higher (P<0.001) *(Table 2)*.

In conclusion, with this study, it has been confirmed that *Babesia* spp. cause DNA damage. It is concluded that further molecular and biochemical studies are needed in the future to better understand the pathogenesis of this infection. This study may set an example for other babesiosis-like piroplasmoses.

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Conflict of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

Datasets analyzed during the current study are available to the corresponding (A. C. Öner) author on reasonable request.

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Author Contributions

ACÖ planned, designed, and supervised the research procedure. AA, FE and ÖOK performed the parasitological analysis, AU and ACÖ performed DNA damage and comet analysis, the statistical analysis, the imaging stage, and the language editing of the final manuscript. AU, FE and ACÖ has revised the manuscript for contents, and approved the final version.

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