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Research Article

Evaluation of Oxidative Stress Parameters in Leptospira Infected Sheep

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ABSTRACT

Leptospirosis is a bacterial infection with zoonotic character which is widespread all over the world, especially in humid and temperate regions and poses a serious threat to public health. In this study, 94 female sheep blood sera sent to Samsun Veterinary Control Institute Directorate with suspicion of leptospirosis were firstly subjected to ELISA (Enzyme Linked Immunosorbent Assay) test for leptospirosis antibodies. total thiol, native thiol, disulphide, native thiol/total thiol, disulphide/native thiol, disulphide/total thiol, malondialdehyde (MDA) levels and adenosine deaminase (ADA) activity were evaluated in the samples.

According to the results of the ELISA test performed in our study, 16 female sheep serum samples were found to be positive for *Leptospira interrogans* ser. hardjo, while 78 samples were found to be negative. To evaluate the effect of leptospirosis on oxidative stress parameters, 15 positive serum samples and 15 negative serum samples taken from healthy sheep were used as control group. According to the results of biochemical analyses, total thiol, native thiol, disulphide, native thiol/total thiol, disulphide/native thiol, disulphide/total thiol values in the *Leptospira* group were not significantly different from those in the control group. However, MDA values and ADA activity in the *Leptospira* group were significantly higher than those in the control group ($P<0.001$). As a result, it was determined that oxidant-antioxidant balance was disturbed in sheep infected with *Leptospira* and oxidative stress occurred as a result. Our findings suggest that MDA and ADA activity can be used as a biomarker in the diagnosis of leptospirosis and may give an idea about the severity of the disease.

Keywords: *Blood sera, Leptospira, Oxidative stress, Sheep.*

Leptospira ile Enfekte Koyunlarda Oksidatif Stres Parametrelerinin Değerlendirilmesi

ÖZET

Leptospirozis tüm dünyada özellikle de nemli ve ılıman bölgelerde yaygın olarak görülen halk sağlığını ciddi boyutta tehdit eden zoonotik karakterli bakteriyel bir enfeksiyondur. Bu çalışmada Samsun Veteriner Kontrol Enstitüsü Müdürlüğüne leptospirozis şüphesi ile gönderilen 94 adet koyun kan serumu öncelikle leptospirozis antikorları yönünden ELISA testine tabi tutuldu. Alınan numunelerde oksidatif stres parametrelerinden toplam tiyol, natif tiyol, disülfit, natif tiyol/toplam tiyol, disülfit/natif tiyol, disülfit/toplam tiyol, MDA düzeyleri ve ADA aktivitesi değerlendirilmiştir.

Çalışmamızda yapılan ELISA testi sonuçlarına göre 16 adet koyun serum örneği *Leptospira interrogans* ser. hardjo yönünden pozitif olarak tespit edilirken, 78 adet örnek ise negatif olarak tespit edilmiştir. Leptospirozisin oksidatif stres parametreleri üzerindeki etkisini değerlendirmek amacıyla 15 adet pozitif serum örneği ve kontrol grubunu oluşturmak amacıyla sağlıklı koyunlardan alınan 15 adet negatif serum örneği kullanıldı. Biyokimyasal analiz sonuçlarına göre; *Leptospira* gruptaki toplam tiyol, natif tiyol, disülfit, natif tiyol/toplam tiyol, disülfit/natif tiyol, disülfit/toplam tiyol değerleri kontrol gruptaki değerler ile karşılaştırıldıklarında anlamlı düzeyde bir değişiklik bulunamamıştır. Fakat *Leptospira* gruptaki MDA değerleri ve ADA aktivitesi kontrol gruptaki değerlere göre anlamlı derecede yüksek bulunmuştur ($P<0.001$). Sonuç olarak *Leptospira* ile enfekte koyunlarda oksidan-antioksidan dengesinin bozulduğu ve bunun sonucunda oksidatif stresin meydana geldiği belirlendi. Bulgularımız MDA ve ADA aktivitesinin leptospirozis tanısında bir biyobelirteç olarak kullanılabileceğini ve hastalığın şiddeti hakkında fikir verebileceğini düşündürmektedir.

Anahtar kelimeler: *Kan serumu, Koyun, Leptospira, Oksidatif stres.*

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Introduction

Leptospirosis is a zoonotic infection that is common worldwide and has serious negative effects on the economies of countries indirectly as well as direct effects on human and animal health (Ellis, 2014; Haake and Levett, 2015). The infection is caused by *Leptospira* species with high genetic diversity (Reller et al., 2014; Picardeau, 2017). *Leptospira* is a Gram-negative aerobic, double-membraned, periplasmic endoflagellum, slender, hook-shaped and highly motile bacterium in the spirochete family (Picardeau, 2017). *Leptospiras*, which are classified as pathogenic, intermediate and saprophytic according to their different levels of pathogenicity in humans and animals (Adler and Moctezuma, 2009) currently have 87 species (LPSN, 2024) and around 300 antigenic serovars (Levett, 2014). Pathogenic *Leptospira* species are the most common causes of leptospirosis worldwide. On the other hand, *Leptospira* species classified as intermediate are potential pathogens that can cause mild infections. Saprophytes are widespread in the environment and are not pathogenic (Guglielmini et al., 2019; Vincent et al., 2019). Leptospirosis remains a serious public health problem in temperate, subtropical and tropical climates due to its wide range of hosts, including humans, domestic and wild animals, which can be reservoir and incidental hosts (Picardeau, 2017; Bertelloni et al., 2019). Reservoir hosts are asymptomatic carriers who excrete *Leptospira* into the environment in urine. The most common cause of leptospirosis is accidental contact with *Leptospira*-infected urine (Adler and Moctezuma, 2009). Therefore, the epidemiology of *Leptospira* is often closely related to the reservoir host of the *Leptospira* serovar (Blasdell et al., 2019). For example, the reservoir hosts of *L. icterohaemorragie* are mice, *L. pomona* are pigs, *L. bratislava* are pigs and horses, *L. canicola* are dogs and *L. hardjo* are cattle and sheep. (Arent et al., 2013; Arent et al., 2015; Arent et al., 2016; Arent et al., 2017a, Arent et al., 2017b). Clinical manifestations of leptospirosis in humans can range from febrile conditions such as mild influenza to severe and fatal symptoms such as internal organ haemorrhages and multiple organ failure. In the acute phase of leptospirosis, patients have symptoms such as renal failure, jaundice, severe pulmonary haemorrhage syndrome (SPHS) and meningitis (Haake and Levett, 2015). Leptospirosis in sheep shows symptoms such as high fever, severe weakness, haemorrhagic and anaemic syndrome in the acute period (Adler and Moctezuma, 2009; Ellis, 2014), in the chronic phase, it causes abortion, premature pup mortality, infertility and severely reduced milk production (Ellis, 1994; Lilenbaum et al., 2009). This zoonotic disease infects approximately 1 million people annually and causes 60000 deaths (Costa et al., 2015). However, the absence of specific symptoms of leptospirosis is thought to cause confusion with other infectious diseases with similar symptoms and thus underdiagnosis. Considering the complex epidemiological cycle of leptospirosis, the development of more sensitive and specific diagnostic methods for the management of acute infections and the detection of subclinically infected reservoir hosts is critical for disease control. Direct detection of active infection is performed by microbiological and molecular methods such as culture, histopathological examination, immune histochemistry and nucleic acid amplification tests (NAAT). Serological methods such as microscopic agglutination test (MAT), ELISA and lateral flow methods are used for the detection of *Leptospira spp.* antibodies. The diagnosis of leptospirosis should be based on potential exposure, clinical presentation, laboratory values such as hemogram and biochemistry, and the results of multiple tests (Nally et al., 2020; Philip et al., 2020). The roles of direct and indirect diagnostic methods in detecting the disease are interdependent. While direct detection methods are more sensitive in the acute phase of infection, indirect, i.e. serological detection methods have higher sensitivity in the chronic phase. A combination of direct and indirect diagnostic methods is necessary to establish the true prevalence of infection in specific host species. For serological and molecular diagnostic methods to be used correctly and effectively, factors such as which *Leptospira* species and serovars will be investigated, the characteristics of the epidemic and the affected species should be well known. In this way, more precise results can be obtained, supported by advanced methods such as whole genome sequencing. Isolation of *Leptospirae* presents technical challenges due to the complexity of non-selective media, the slower growth of *Leptospirae* compared to other organisms that may contaminate the media, and the need for dark field microscopy to reliably assess culture positivity. Recently developed selective media in culture approaches (Chakraborty et al., 2011) and suitable media for sensitive organisms have partly overcome these difficulties (Hornsby et al., 2020).

New diagnostic tests are needed for rapid and accurate diagnosis of acute infections. Initiating interventions such as antibiotics and supportive treatment early in the disease is critical in treating the infection. Improved diagnostic methods for surveillance and control in animals are needed to assess the effectiveness of eradication of renal tubular or genital infections following antimicrobial therapy (Ellis et al., 1985). For this reason, in recent years, many researchers have been investigating the usefulness of oxidative stress parameters in the diagnosis of infectious and non-infectious diseases. Oxidative stress plays an important role in the pathophysiology of many diseases that threaten animal health such as sepsis, mastitis, acidosis, ketosis, enteritis, pneumonia, respiratory and joint diseases (Lykkesfeldt and Svendsen, 2007; Celi, 2011). Microorganisms such as bacteria, viruses (Valyi-Nagy and Dermody, 2005) and parasites (Stocker et al., 1985) can increase the production of reactive oxygen radicals (ROS) by disrupting metabolic processes in host cells. Leptospirosis is a toxin-mediated infection that causes lipid peroxidation because of the cytotoxic effect of the membrane lipopolysaccharide in the structure of *Leptospira* bacteria on the host cell (Kim et al., 1997).

Therefore, oxidative stress is thought to play a role in the pathogenesis of leptospirosis.

The aim of this study was to evaluate the effect of leptospirosis on oxidative stress parameters in sheep.

Materials and Methods

Calculation of Sample Size

In this study, the sample size was calculated using the formula proposed by Daniel W.W. based (Daniel, 1999) on a 95% confidence level, an estimated prevalence (P) of 6%, and a 5% margin of error (d = 0.05), the minimum required sample size was determined to be 92.

Study Material

The material of our study consisted of 94 female sheep blood serum samples sent to Samsun Veterinary Control Institute Directorate with suspicion of Leptospirosis (showing haematuria and icterus findings during clinical examination) between 2023-2024. This study was carried out with the permission of the Local Ethics Committee for Animal Experiments of Samsun Veterinary Control Institute Directorate with the letter dated 28.01.2025 and numbered 19572899/031-92.

Serological Analysis

Serum samples were tested for *Leptospira* with a commercial antibody ELISA test kit (BT LAB Sheep *Leptospira* IgG Antibody Test Kit). The test kit and serum samples were kept at room temperature for 30 minutes before starting the test. 50 µl of negative control was added to the negative control well, 50 µl of positive control was added to the positive control well, 40 µl of sample diluent and then 10 µl of serum sample were added to the wells to be used for samples and mixed thoroughly. The prepared plate was incubated at 37°C for 30 minutes. After the first incubation, the plate was washed 5 times with washing solution. After washing, 50 µl HRP (Horse Radish Peroxidase) conjugate was added to each well of the plate and the plate was incubated again at 37°C for 30 minutes. After incubation, washing was performed again. 50 µl of substrate solution A and 50 µl of substrate solution B were added to each well of the plate and incubated at 37°C for 10 min in the dark. After this final incubation of the assay, 50 µl of stop solution was added to each well of the plate and the optical density (OD) values of the wells were read at 450 nm on an ELISA reader (Mindray MR-96A). The OD values obtained were evaluated as positive or negative according to the evaluation criteria specified in the test kit below,

Cutoff Value=Negative Control value + 0.15

OD Value<Cutoff value is negative,

OD Value≥Cutoff value was considered positive.

Biochemical Analysis

Native thiol (Rel Assay) and total thiol (Rel Assay) oxidative stress parameters in serum samples were measured using colorimetric test kits according to the procedure recommended in the kit. The spectrophotometric method described by Erel and Neselioglu (2014) was used for measurement. In this method, the disulphide bonds formed because of oxidation were converted to optional thiol groups by $NaBH_4$/sodium borohydride. Unused sodium borohydride was removed by binding with formaldehyde. The amount of native thiol and the amount of total thiol formed by the reduction of disulphide bonds were measured with DTNB (5,50-dithiobis-(2 nitrobenzoic acid) chromogen at 412 nm in ELISA plate reader. When calculating the amount of disulphide, native thiol was subtracted from total thiol and half of the difference was taken.

Malondialdehyde (MDA) analysis was measured by the method reported by Yoshioka et al (Yoshioka et al., 1979). The lipid content in the test thiobarbituric acid (TBA) reaction was carried out by the formation of a stable red-pink colour with a minimum peak at 535 nm when heated at low pH and in the presence of TBA. Red-pink colour, chromogen formed by the combination of an MDA molecule and two TBA molecules. Some of the MDA was formed during peroxidation, the majority was formed by the breakdown of LPO during the heating phase after acidification of the medium. The test was performed spectrophotometrically. Adenosine deaminase (ADA) activity was measured by Giusti method (Giusti, 1974). Using the ammonium ion released from adenosine by enzyme action, the formation of indophenol complex because of Bertholet Reaction and measurement of this complex at 623 nm in a spectrophotometer device was carried out according to the principle.

Statistical Analysis

All statistical calculations of the data were performed using SPSS statistics 27.0 (IBM SPSS Inc, Chicago, IL, USA) package programme. Shapiro-Wilk test was used to evaluate whether the data were normally distributed. It was determined that the data showed normal distribution. T test was used to determine the differences between groups in all data. $P<0.05$ was considered statistically significant.

Results

As a result of the ELISA test performed on 94 female sheep blood sera, 16 (17.02%) serum samples were positive for *L. interrogans ser. hardjo* and 78 (82.98%) were negative. As a result of the biochemical analyses performed on 15 *Leptospira* positive serum samples and 15 *Leptospira* negative control group; Total thiol, native thiol, disulphide, native thiol/total thiol, disulphide/native thiol, disulphide/total thiol values in the *Leptospira* group were not significantly different from the values in the control group. However, MDA activity and ADA in the *Leptospira* group were significantly higher than the values in the control group ($P<0.001$). The positivity and negativity of the samples are given in Table 1, oxidative stress parameter results are given in

Table 1. Positive and negative status of the samples

Total Number of Samples	Number of Positive Samples	Number of Negative Samples
94	16 (17.02%)	78 (82.98%)

Table 2 and measurement graphs of these parameters are given in Figures 1, 2 and 3.

Table 2. Oxidative stress parameter values of *Leptospira* positive and control groups*

Parameters	Healthy Group	Leptospirosis Group	P values
Total thiol (µmoL/L)	246.63±64.06	264.57±49.98	0.449
Native thiol (µmoL/L)	112.81±29.20	99.98±33.60	0.338
Disulphide (µmoL/L)	66.91±30.59	82.34±24.07	0.180
Native thiol/Total thiol (%)	47.98±17.00	38.04±11.19	0.097
Disulphide/Native thiol (%)	65.32±36.84	91.74±39.57	0.111
Disulphide/Total thiol (%)	26.00±8.50	30.97±5.59	0.097
Malondialdehyde (µmol/mL)	0.96±0.20	1.91±0.53	<0.001
Adenosine deaminase (U/L)	6.45±1.21	10.77±2.72	<0.001

*Data are presented as mean ± standard error of the mean (SEM).

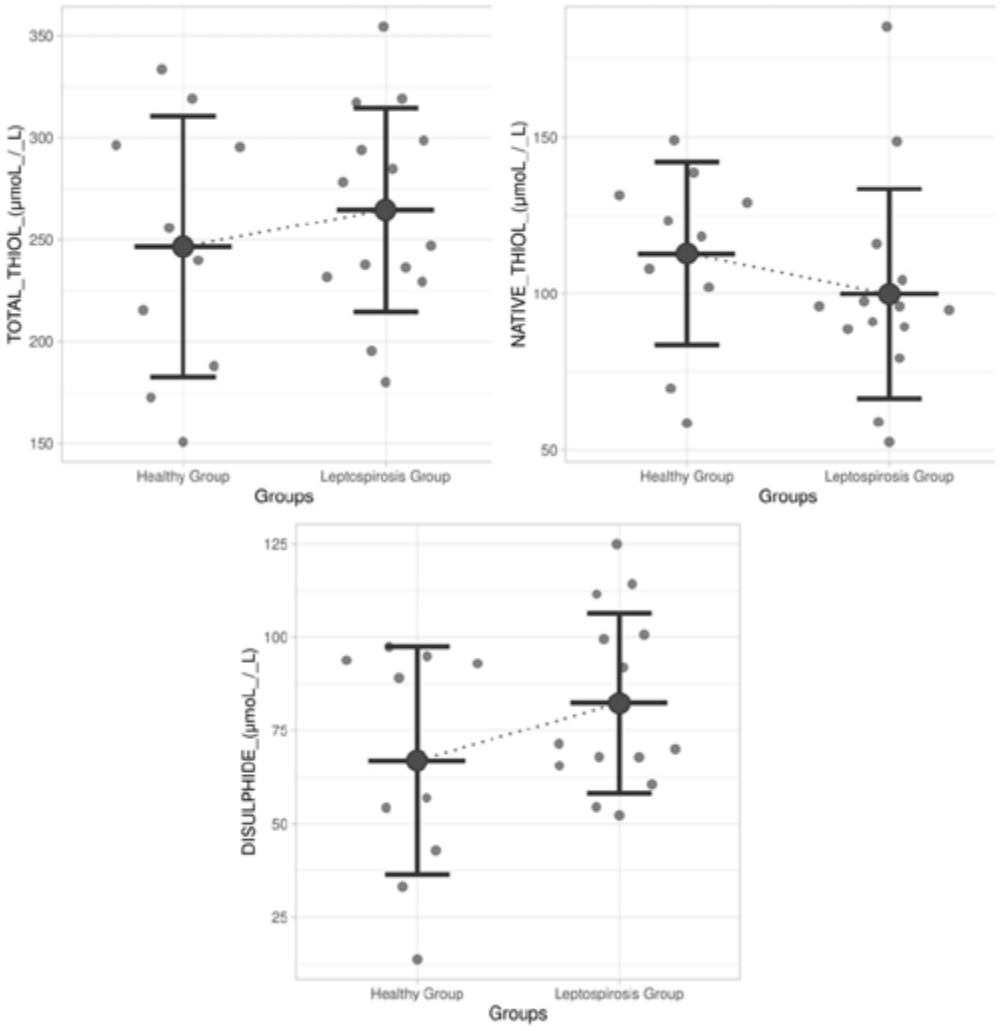

Figure 1. Serum native thiol, total thiol and disulphide values

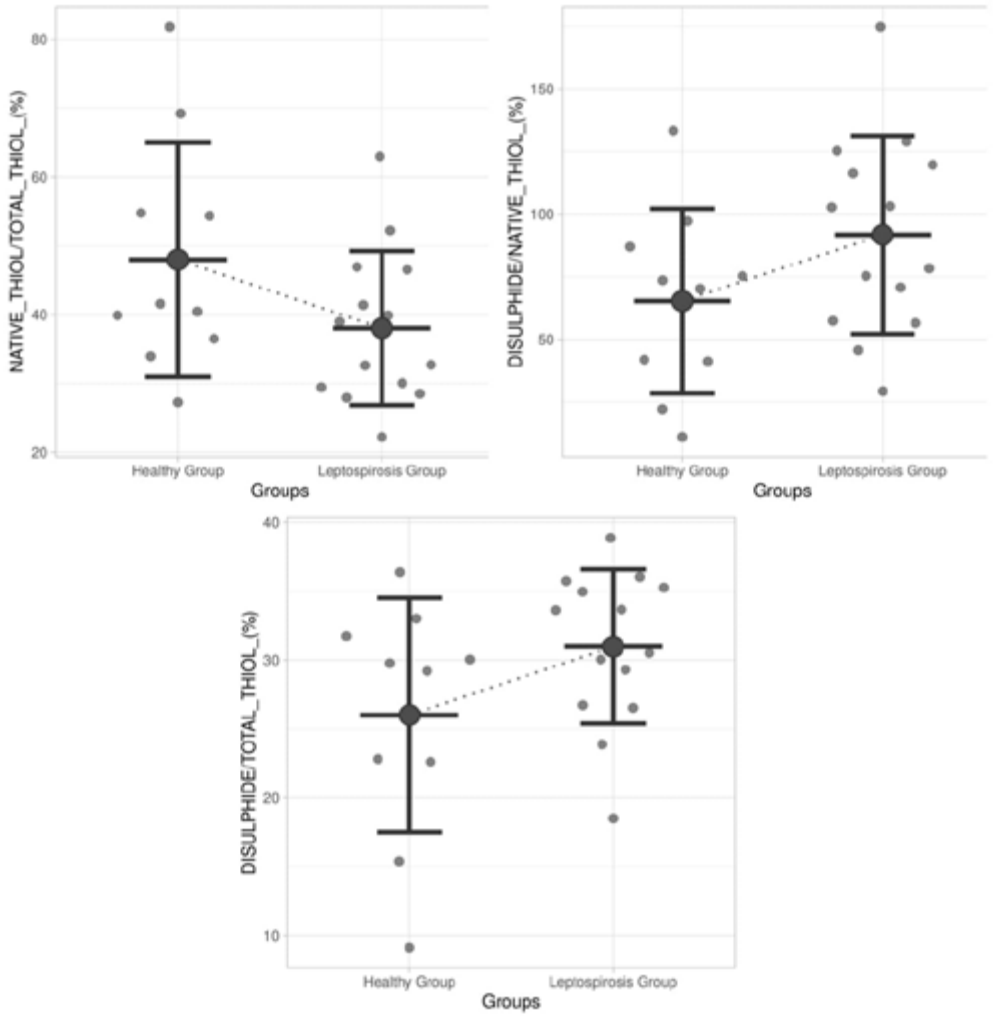

Figure 2. Serum native/total thiol (%), disulphide/native thiol and disulphide/total thiol (%) values

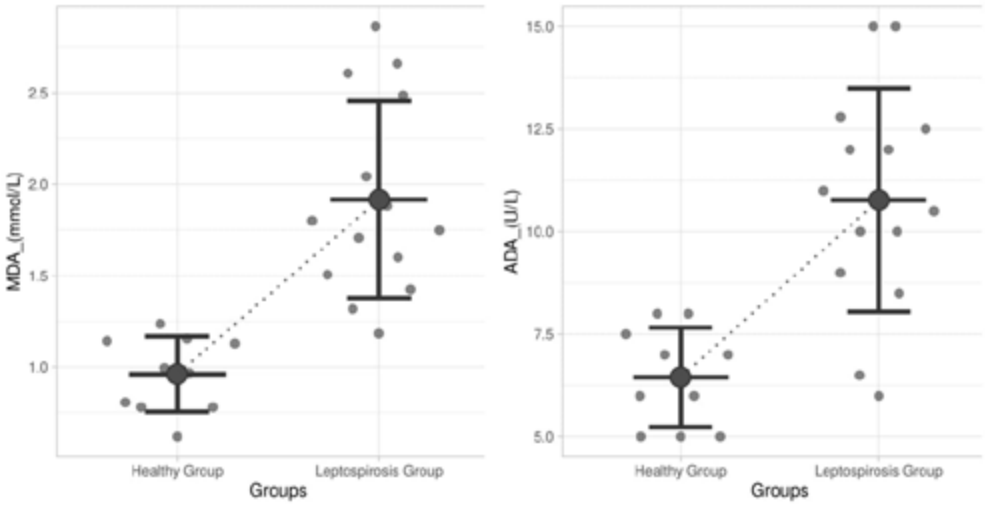

Figure 3. Serum MDA and ADA values

Discussion

The incidence of leptospirosis, which has zoonotic character and seriously threatens human and animal health in the world and in our country, varies from country to country and from region to region. There are studies on the prevalence and seropositivity of leptospirosis in our country. The seropositivity rate reported in serological studies in cattle is between 14% and 38.6%. Although there are not many serological studies on leptospirosis in sheep in our country, the rates reported in a few serological studies are between 2.9% and 8% (Harran et al., 2022). In our study, the seropositivity rate was found to be 17.02%. Our seropositivity rate was higher than other studies in our country. This may be because the mate-

rials used in our study were obtained from sick sheep showing symptoms of leptospirosis. The seropositivity rate reported in serological studies in cattle in European countries is between 0% and 100% (Sohm et al., 2023). In serological studies conducted in different countries around the world, it has been reported that the seropositivity rate of leptospirosis in sheep is between 0% and 60.7% (Antoniolli et al., 2024). It is thought that the reason for such differences between the results of the studies may be differences such as climate, geographical region, sampling times, selection methods of the samples and the reasons for taking the samples.

Diagnostic methods of bacterial, viral, fungal and tumoral diseases are updated every day. Oxidative stress parameters have started to be used as an important guiding marker for the diagnosis and prognosis of these diseases. There are many studies proving that oxidative stress parameters can be used as biomarkers in the diagnosis of leptospirosis and in understanding the extent of the damage caused by this disease in tissues and organs and consequently in determining the treatment method. The most researched of these parameters is MDA, which is formed because of lipid peroxidation and has mutagenic and toxic effects (Esterbauer et al., 1990). Once formed, MDA can be degraded by different enzymes, particularly mitochondrial aldehyde dehydrogenase, or it can interact with proteins and nucleic acids to form various compounds that damage DNA-protein cross-links and biomolecules (Marnett, 1999; Voitkun and Zhitkovic, 1999). MDA has been used as a biomarker to determine oxidative stress in various biological samples such as blood, serum and urine from sick humans and animals affected by a wide range of diseases including non-infective diseases such as cancer, cardiovascular, pulmonary, neurodegenerative diseases and infective diseases such as brucellosis, tuberculosis and leptospirosis (Merendino et al., 2003; Del Rio et al., 2005). The detection of end products such as MDA in infective diseases proves that lipid peroxidation has an important role in the prognosis of such diseases (Busch and Binder, 2016). The membrane polysaccharide in the structure of *Leptospira* bacteria causing leptospirosis causes lipid peroxidation because of its cytotoxic effect on the host cell (Kim et al., 1997) When considered, it is evaluated that MDA may have the potential to be an important marker in the diagnosis of leptospirosis. For this reason, there are various studies evaluating MDA levels in animals infected with leptospirosis. In previous studies, MDA levels were found to be statistically significantly higher in the *Leptospira*-infected group compared to the control group, as in our study (Erdoğan et al., 2008; Gazioğlu and Güvenç 2015; Niroomandi et al., 2022). Other studies and the findings obtained in our study strengthen the opinion that MDA can be used as a biomarker in the diagnosis of leptospirosis.

Adenosine deaminase is a very important enzyme of purine metabolism. Its function in purine metabolism is to irreversibly reduce adenosine and deoxyadenosine to inosine and deoxyinosine, respectively (Franco et al., 1997). ADA is found in all cell types, but higher ADA activity has been found in lymphoid tissues, thymus and peripheral lymphocytes. In addition, another important function of ADA is the differentiation, maturation and proliferation of T lymphocytes (Franco et al., 1997; Cordero et al., 2001). Considering the damage caused by *Leptospira* in the blood vessels and cells of the host, we believe that ADA can be used as a biomarker in the diagnosis of leptospirosis. However, there are not many studies evaluating the activity of ADA in leptospirosis infection. In the literature review conducted within the framework of this study, 2 studies evaluating the activities of ADA in leptospirosis infection were found. The first of these was the study conducted in cattle by Atakişi et al. In this study, ADA activity in the *Leptospira*-infected group was found to be statistically higher than in the control group as in our study (Atakişi et al., 2014). However, in the experimental study of Tonin et al. (2012) on mice, ADA activity in the infected group was found to be statistically lower than in the control group, contrary to the results of our study and the study of Atakişi et al (2014). Although ADA activity was found to be lower in the infected group in one of the three studies in contrast to the others, the statistically different result from the control group suggests that ADA may be a potential biomarker in the diagnosis of this disease. However, we believe that more studies should be conducted on the evaluation of leptospirosis infections of ADA.

Thiol-disulphide balance plays a crucial role in antioxidant defence, immune response, regulation of enzyme activity and apoptosis (Valko et al., 2006). There are studies evaluating thiol disulphide balance in infective diseases of viral and bacterial origin such as Crimean-Congo haemorrhagic fever, acute tonsillopharyngitis and brucellosis. In these studies, it was reported that thiol-disulphide balance was disturbed, and this parameter could be used as a biomarker in the diagnosis of these infective diseases. However, no study was found in which thiol-disulphide balance was evaluated in *Leptospira* infection. Although we could not detect a significant difference in thiol-disulphide levels in the control and infective groups, we believe that further studies should be conducted in this field.

Conclusion

Scientific studies and the findings obtained in this study showed that MDA and ADA can be used as biomarkers in the diagnosis of *Leptospira* infections. Studies evaluating oxidative stress parameters in *Leptospira* infections were mostly performed in cattle and to a lesser extent in horses and dogs. In the literature review conducted within the framework of this study, no study evaluating oxidative stress parameters in *Leptospira* infections in sheep was found. For this reason, we believe that scientific studies should be carried out in this field. In addition, we could not find any study evaluating the thiol-disulphide balance in *Leptospira* infections other than our study. We think that the evaluation of this important parame-

ter, which has been proven to be used as a biomarker in the diagnosis of infective and non-infective diseases, in further studies will contribute to the literature.

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Author Contribution Statement

All authors are responsible for the entire content of this article and have approved its submission.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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