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Investigation of Oxidative Stress Parameters in Cattle Infected with *Mycobacterium avium subsp. Paratuberculosis*

Mycobacterium avium subsp. paratuberculosis ile Enfekte Sığırlarda Oksidatif Stres Parametrelerinin İncelenmesi

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ABSTRACT

Paratuberculosis is a zoonotic disease caused by Mycobacterium avium subsp. paratuberculosis (MAP) in cattle. MAP may cause the formation of reactive oxygen species (ROS) by increasing the release of proinflammatory cytokines in the host. Due to the increase in ROS, the oxidant-antioxidant balance may be disrupted and oxidative stress may occur. The aim of the study was to determine the oxidative stress parameters in cattle infected with paratuberculosis. For this purpose, 15 cattle sera that were positive for paratuberculosis and 15 clinically healthy 30 cattle sera were used as the control group. In the samples taken, oxidative stress parameters such as total antioxidant capacity (TAS), total oxidant capacity (TOS), oxidative stress index (OSI), native thiol, total thiol and disulphide levels were evaluated. According to our study results, TOS (21.911±11.80), OSI (37.99±21.40), total thiol (1836.671±877.06) and disulphide (715.420±395.32) values in the paratuberculosis group were significantly higher than TOS (8.538±5.18), OSI (10.24±7.23), total thiol (823.809±289.86) and disulphide (197.936±131.70) values in the control group (*P*<.001). The TAS (0.588±0.14) value in the paratuberculosis group was significantly lower than the TAS (0.952±0.26) value in the control group (*P*<.001). No significant difference was found between the two groups in terms of native thiol levels (*P*>.05). As a result, it was determined that the oxidant-antioxidant balance was disrupted and oxidative stress occurred in MAP infected cattle. Therefore, it was concluded that oxidative stress parameters can be used as biomarkers in the diagnosis and treatment of the disease.

Keywords: Cattle, oxidative stress, paratuberculosis.

ÖZ

Paratüberküloz sığırlarda Mycobacterium avium subsp. paratuberculosis (MAP) tarafından oluşturulan zoonotik bir hastalıktır. MAP konakçıda proinflamatuar sitokinlerin salımını artırarak reaktif oksijen türleri (ROS) oluşumuna neden olabilir. ROS artışına bağlı olarak da oksidan antioksidan denge bozulabilir ve oksidatif stres ortaya çıkabilir. Çalışmanın amacı paratüberküloz enfekte sığırlarda oksidatif stres parametrelerinin belirlenmesidir. Bu amaçla çalışmada paratüberküloz yönünden pozitif tespit edilmiş 15 adet sığır serumu ve kontrol grubu olarak 15 adet klinik açıdan sağlıklı toplam 30 adet sığır serumu kullanılmıştır. Alınan numunelerde oksidatif stres parametrelerinden total antioksidan kapasite (TAK), total oksidan kapasite (TOK), oksidatif stres indeksi (OSİ), native thiol, total thiol ve disülfid düzeyleri değerlendirilmiştir. Çalışma sonuçlarımıza göre paratüberküloz grupta TOK (21,911±11,80), OSİ (37,99±21,40), total thiol (1836,671±877,06) ve disülfid (715,420±395,32) değerleri kontrol grubundaki TOK (8,538±5,18), OSİ (10,24±7,23), total thiol (823,809±289,86) ve disülfid (197,936±131,70) değerlerine göre anlamlı düzeyde yüksek belirlenmiştir (*P*<,001). Paratüberküloz grubunda ki TAK (0,588±0,14) değeri ise kontrol grubunda ki TAK (0,952±0,26) değerine göre anlamlı düzeyde düşük belirlenmiştir (*P*<,001). Native thiol düzeyleri açısından her iki grup arasında anlamlı bir fark belirlenememiştir (*P*>,05). Sonuç olarak MAP enfekte sığırlarda oksidan antioksidan dengenin bozulduğu ve oksidatif stresin ortaya çıktığı belirlenmiştir. Bu nedenle hastalığın tanı ve tedavisinde oksidatif stres parametrelerinin biyobelirteç olarak kullanılabileceği kanaatine varılmıştır.

Anahtar Kelimeler: Oksidatif stres, paratüberküloz, sığır.

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INTRODUCTION

Paratuberculosis is a disease causing granulomatous enteritis, usually in cattle, sheep and goats, characterised by chronic diarrhea. The etiology of this disease is *Mycobacterium avium subsp. paratuberculosis* (MAP).[1] *Mycobacterium avium subsp. paratuberculosis* is an acid-fast bacillus that causes granulomatous, incurable enteritis progressing to severe cachexia and death. The disease is transmitted through infected drinking water, dry grasses, carrier animal droppings, and contaminated environments. In addition, housing calves together with the mother after birth and breastfeeding also play an important role in the transmission of infection. After ingestion, MAP settles in the lymphoid tissue associated with the intestine. *Mycobacterium avium subsp. Paratuberculosis* requires high concentrations of iron for development. There is a high amount of iron in tissue macrophages located in the ileocaecal intestinal passage.[2] In this way, MAP causes granulomatous enteritis and thickening in the ileocaecal duct.[3] In addition, hypertrophy and edema of the lymph nodes in the mesenteric region are observed. Microscopically, the disease is characterized by varying degrees of chronic granulomatous enterocolitis, regional lymphangitis, and lymphadenitis.[4] Even subclinical infection significantly reduces the performance of animals and leads to direct and indirect economic losses in the cattle industry.[5] The main economic losses of the disease are; decrease in milk yield and quality, decrease in live weight gain, infertility problems and increased susceptibility to other chronic diseases.[6] It has also been shown that MAP can be transmitted to humans and play a role in the etiology of a disease with clinical symptoms similar to paratuberculosis and has been named Crohn's disease (CD), which causes chronic inflammation of the intestine in humans.[7] Therefore, paratuberculosis is important in terms of public health as well as the economic losses it causes. The source of transmission of MAP bacteria to humans is meat, meat products, milk, dairy products, and drinking water contaminated with feces of paratuberculosis-infected animals. Since the transmission of MAP is foodborne, it is important for food safety. Crohn's disease is a chronic inflammatory disease that affects the gastrointestinal tract of humans from the mouth to the anus. The intestinal segment narrows and thickens and the accompanying mucosal deep ulcers and fissures give a cobblestone appearance.[8] The general symptoms of the disease are abdominal pain, diarrhea, and weight loss. Diarrhea is found in approximately 70% of patients with Crohn's disease. Weight loss may develop due to malabsorption and anorexia.[9] It is important to carry out detailed research due to reasons such as the fact that MAP is accepted as a pathogen in humans, it is more resistant to the temperature-time degrees of pasteurization than other pathogens, and the possibility of transmission with foods obtained from infected animals.[10] MAP is also present as an intracellular pateogen in macrophages of infected cattle. It decreases the phagocytosis ability of macrophages by preventing the pH in lysosomes from decreasing. It is also resistant to degradation even in lysosome.[11] In contrast, the body's primary defence mechanism is the induction of apoptosis of infected macrophages through a tumour necrosis factor-a (TNF-a)-dependent mechanism.[12,13] However, when apoptosis is not sufficient to clear infected cells, it may cause the cell to lose its membrane integrity and transform into secondary necrotic cells. This may result in systemic inflammation, oxidative stress and the formation of reactive oxygen species (ROS).[14,15] Reactive oxygen species are important defense substances involved in the immune response against pathogens.[16] However, excessive increases in ROS can cause oxidative stress (OS) by damaging the organism. Antioxidant enzymes (AE) are produced in the organism in order to neutralise OS. However, tissue damage may develop as a result of disruption of oxidant and antioxidant balance.[17] Since the response to OS is both an indicator of the activation of the immune system and an indicator of the ability to compensate for infection-induced damage, it is used as a biomarker in many studies. Considering all these situations, further research should be carried out for the rapid detection of MAP in meat and meat products, milk, and dairy products and new methods for diagnosis should be developed. Because the diagnosis of the disease is important for both economic, human, and food health. In this study, it was aimed to determine the levels of total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), native thiol, total thiol, and disulphide as biomarkers of oxidative stress parameters in cattle with serologically detected paratuberculosis.

MATERIALS AND METHODS

This study was carried out on the samples received for routine analyses from livestock farms in Amasya, Tokat, and Samsun provinces within the responsibility area of Samsun Veterinary Control Institute between 2023-2024. Paratuberculosis vaccination was not performed in the enterprises where the samples were received. In the study, 15 cattle blood samples that were positive for paratuberculosis and 15 clinically healthy cattle blood samples that were negative for paratuberculosis, totally of 30 cattle blood samples, were used as the control group. Serum samples received at the institute were stored at -20 °C until the analyses were performed.

Serum samples were tested for paratuberculosis using a

commercial antibody ELISA kit (IDEXX Mycobacterium paratuberculosis Antibody Test Kit). The test kit and samples were allowed to reach room temperature. The serum samples to be analysed, positive and negative control sera were diluted 1/20 using Dilution Buffer No12 in a sterile U-bottom microplate and pre-incubated for 15 min at 18-22 °C in a microplate shaker. After preincubation, 100 µl of all sera were added to the wells of the test kit plate. The plate was incubated in a shaker for 45 minutes at 18-22 °C. At the end of the incubation period, the plate was washed 3 times with washing solution. Anti-ruminant HRPO conjugate was diluted 1/100 in Dilution Buffer No1 and 100 µl was added to each well of the plate. It was incubated again at 18-22 °C for 30 minutes. At the end of the incubation period, the washing process was repeated. TMB-substrate solution was added 100 µl to each well of the plate and incubated at 18-22 °C in the dark for 10 minutes. At the end of incubation, 100 µl of stop solution was added to the wells and the reaction was terminated. Optical density (OD) values of the wells were read at 450 nm on an ELISA reader (Mindray MR-96A). The OD values obtained were placed in the formula below and the results were calculated.

$$\boldsymbol{Result}\ (\%) = \frac{\mathbf{ODsample} - \boldsymbol{ODnegative}}{\mathbf{ODpositive} - \mathbf{ODnegative}}\ \boldsymbol{x}\ \mathbf{100}$$

The results obtained according to the formula were evaluated as 55% and above positive, 45-55% suspect, and less than 45% negative. Results in the suspect range were not included in the study.

The study was conducted by the decision of the ethics committee numbered 2024/04, 19572899/031-85, taken by the "Ethics Committee Directive" of the Ministry of Agriculture and Forestry Samsun Veterinary Control Institute Animal Experiments Local Ethics Committee.

Biochemical Analyses

Total antioxidant status (Rel Assay Diagnostics, RL0017, Turkey), TOS (Rel Assay Diagnostics, RL0024, Turkey), native thiol (Rel Assay Diagnostics, RL0185, Turkey), and total thiol (Rel Assay Diagnostics, RL0192, Turkey) oxidative stress parameters were measured using colorimetric test kits according to the procedure recommended in the kit. The measurements of the kits used were performed on an ELISA plate reader (Tecan Infinite F50, Switzerland). Oxidative stress index was calculated using TAS and TOS data and disulphide was calculated using native thiol and total thiol parameters.

Total thiol and native thiol levels are determined using colorimetric test kits. The difference between the obtained values indicates the dynamic disulphide content. SS/SH+SS, SS/SH and SH/SH+SS ratios are calculated. These ratios show how much space disulphide bonds occupy. The methodology allows to analyze the balance of thiol and disulphide groups.

Statistical Analyses

Statistical analyses were performed using the SPSS Statistic 27 program. To determine the normality of the distribution, skewness, kurtosis values, and the Shapiro-Wilk test were used. For normally distributed groups, t-test was used to compare the groups. P values below 0.05 were considered significant. Since the data obtained showed normal distribution, the Pearson test was used to determine the correlation between the groups.

RESULTS

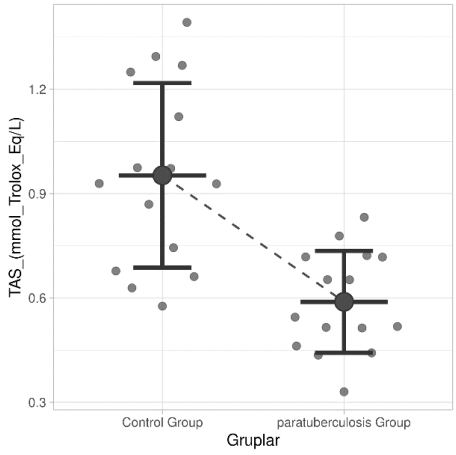
In the study, the values of some oxidative stress parameters were determined in blood samples taken from MAP-positive cattle and MAP-negative cattle. Oxidative stress values of control and MAP-infected cattle are given in Table 1.

Table 1. TAS, TOS, OSI, Total thiol, Native thiol, Disulphide, Native thiol/Total thiol (%), Disulphide/Native thiol (%), and Disulphide/Total thiol (%) levels (mean ± SD) in control and MAP-infected cattle.

Parameters	Control Group	Paratuberculosis Group	*P*
TAS (mmol Trolox Eq/L)	0.952±0.26	0.588±0.14	< .001
TOS (µmol H2O2 Eq/L)	8.538±5.18	21.911±11.80	< .001
OSI (AU)	10.24±7.23	37.99±21.40	< .001
Total thiol (µmoL / L)	823.809±289.86	1836.671±877.06	< .001
Native thiol (µmoL / L)	427.937±255.77	405.830±142.75	> .05
Disulphide (µmoL / L)	197.936±131.70	715.420±395.32	< .001
Native thiol/Total thiol (%)	51.94±26.00	22.09±15.81	< .01
Disulphide/Native thiol (%)	46.25±32.93	176.28±120.71	< .001
Disulphide/Total thiol (%)	24.02±13.00	38.95±7.90	< .01

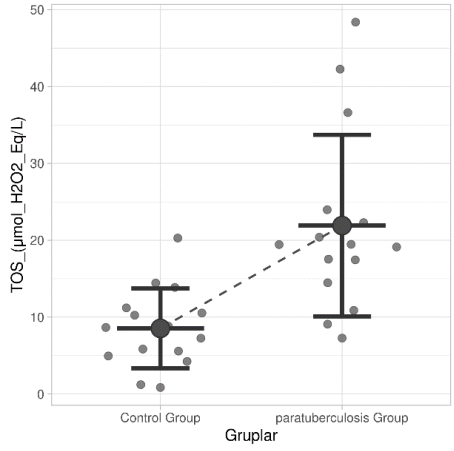
TAS, total antioxidant status; TOS, Total oxidant status; OSİ, Oxidative stress index.

Serum TAS, TOS, and OSI levels ($P < .001$) of the control group and paratuberculosis group are given in figure 1, figure 2, and figure 3.



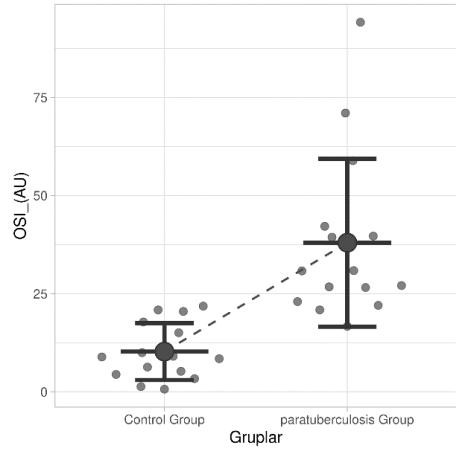
TAS: total antioxidant status

Figure 1. Serum TAS levels



TOS: total oxidant status

Figure 2. Serum TOS levels



OSI: oxidative stress index

Figure 3. Serum OSI levels

Serum total thiol levels ($P < .001$), native thiol ($P > .05$), and disulphide levels ($P < .001$) of the control group and paratuberculosis group are given in figure 4, figure 5, and figure 6.

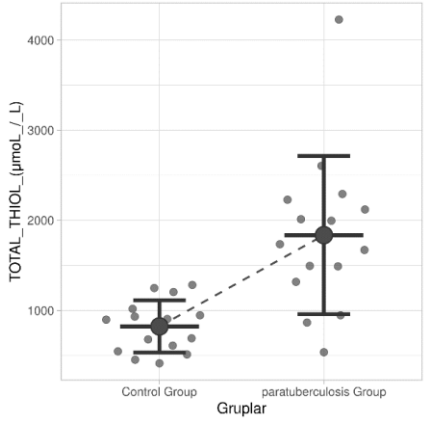


Figure 4. Serum total thiol levels

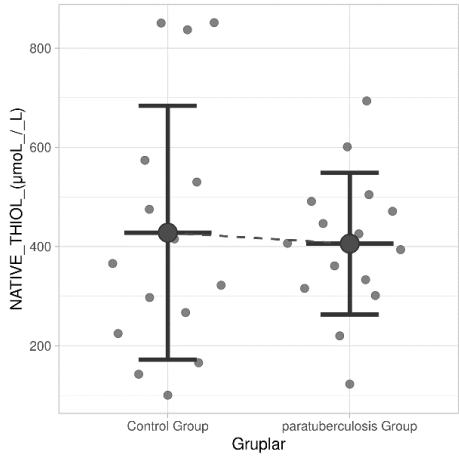


Figure 5. Serum native thiol levels

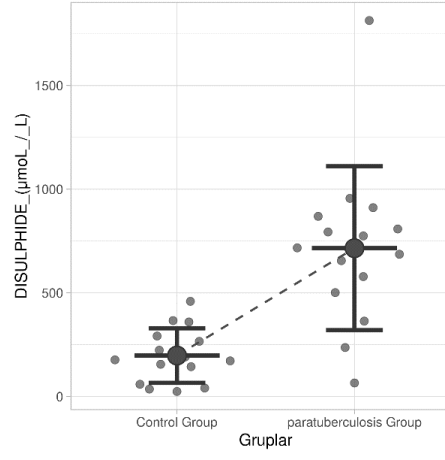


Figure 6. Serum disulphide levels

Serum native thiol/total thiol (%) levels ($P < .01$), disulphide/native thiol (%) levels ($P < .001$), disulphide/total thiol levels ($P < .01$) of the control group and paratuberculosis group are given in figure 7, figure 8, and figure 9.

According to Pearson correlation analysis, TAS was negatively correlated with TOS and OSI, while OSI and TOS were positively correlated ($P < .05$). There was also a positive correlation between total thiol, TOS, OSI, and disulphide ($P < .05$). Disulphide/native thiol (%) ratio was positively correlated with TOS, total thiol, disulphide, disulphide/total thiol (%) ratio and negatively correlated with native thiol/total thiol (%) ratio ($P < .05$).

Disulphide/total thiol (%) ratio was positively correlated with total thiol, disulphide, disulphide, native thiol and native thiol/total thiol (%) ratios ($P < .05$). Native thiol/total thiol (%) ratio was negatively correlated with total thiol, disulphide, disulphide/total thiol (%), disulphide/native thiol (%) ratio and positively correlated with native thiol ($P < .05$).

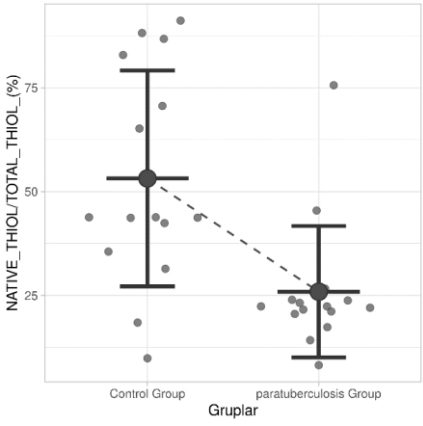


Figure 7. Serum Native thiol/total thiol (%) levels

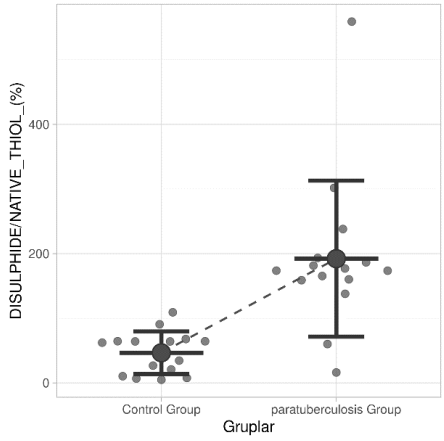


Figure 8. Serum Disulphide/Native thiol (%) levels

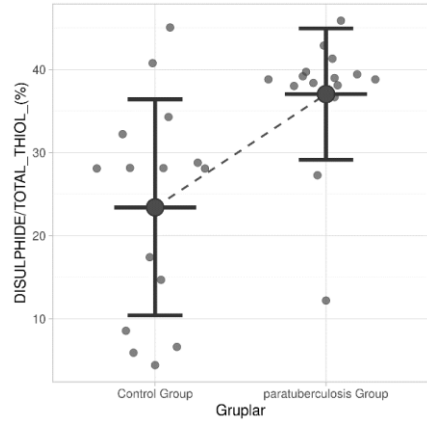


Figure 9. Serum Disulphide/total thiol (%) levels

DISCUSSION

Paratuberculosis is a granulomatous enteritis characterised by chronic diarrhea caused by *Mycobacterium avium subspecies paratuberculosis* infection. It mostly affects ruminants and animal welfare, raises public health concerns, and can cause direct and indirect economic losses.[18] Increased release of ROS is reported in bacterial diseases such as paratuberculosis. Free radicals or ROS are released from dendritic cells, neutrophils, and macrophages in response to an inflammatory stimulus.[19] The ROS produced must be kept in balance by the antioxidant system. If the oxidant-antioxidant balance is disrupted as a result of an increase in ROS levels in cells, oxidative stress, which has an important role in the pathophysiology of diseases, may occur.[20] Oxidative stress results from a disturbance of the balance between the production of reactive oxygen or nitrogen derivatives and the organism's defence capability.[21] During infection, defence cells produce large amounts of ROS and RNS in order to clear pathogens. However, these biochemical products may result in the triggering of a pathological process affecting healthy structures as well as pathogens.[22] Therefore, the oxidative response can be used as a biomarker indicating both the presence of the pathogen[23] and the onset of defence against pathogens.[24] For this purpose, we aimed to determine how TAS, TOS, native thiol, and total thiol parameters, which are oxidative stress markers, change and whether oxidative response occurs in MAP-infected cattle.

In this study, TOS, OSI, total thiol, and disulphide levels were significantly higher in MAP-infected cattle, while TAS level was significantly lower in MAP-infected cattle compared to the control group. There was no significant difference in native thiol levels between both groups. Merhan et al.[25] determined that MDA and NO levels, which are oxidative stress parameters, were significantly increased in the MAP-infected group in a study conducted in 15 cattle infected with MAP and 15 clinically healthy cattle.[25] Cenesiz et al.[26] evaluated oxidative stress parameters such as MDA, SOD, and GSH-Px in another study and determined that while MDA level increased significantly in the MAP-infected group, there was no significant change in SOD and GSH-Px enzyme activity levels.[26] Akyuz et al.[27] reported that oxidative stress developed in cattle with subclinical paratuberculosis and serum MDA level was significantly higher compared to the control group.[27] Balıkcı and Gurdogan[28] found that SOD, GSH-Px, and GSH levels decreased significantly in clinical and subclinical groups compared to the control group in a study conducted in sheep with paratuberculosis.[28] El-Deeb et al.[29] in a study conducted in paratuberculosis-infected dromedary camels, MDA level of the paratuberculosis-infected group increased while SOD, CAT, and reduced glutathione enzyme activities decreased. As a result of this situation, it was determined that oxidative stress developed.[29] In the presented study was similar to other studies and it was determined that TOS and OSI levels increased while TAS level decreased in MAP-infected cattle. This situation suggests that free radical formation increases

as a result of infection and accordingly, oxidative stress develops by disrupting the oxidant-antioxidant balance. Because tissue damage, inflammation, and infections cause free radical formation by activating phagocytic cells which have an important role in host defense.[30] It has been reported that oxidative stress occurs by disruption of oxidant-antioxidant balance especially in bacterial and viral diseases. The decrease in TAS activity in the study indicates that the antioxidant defense system is insufficient due to increased oxidative stress due to free radical formation. In addition, it was determined that disulphide level increased in the MAP-infected group. As a result of the literature review, no studies evaluating native total thiol levels in cattle with paratuberculosis were found. However, it was determined that native thiol, total thiol, and disulphide levels changed in some infected diseases in cattle.[31–34] The reason for this situation is that the increase in proinflammatory cytokines due to the increase in infection may cause an increase in ROS, which may cause an increase in disulphide levels by binding native and total thiols with reversible disulphide bonds to increasing oxidants. To maintain oxidative balance in the organism, the conversion of thiol groups into disulphide bonds increases. Thiols can be converted into disulphide bond structure by oxidation by oxidant molecules. This transformation is an early indicator of protein oxidation under oxidative stress. Disulphide formation reflects antioxidant status and redox status, indicating oxidative stress. The resulting disulphide bond is converted back to thiol groups, thus ensuring thiol-disulphide balance. In biological organisms, thiol-disulphide balance can be used as a biomarker to evaluate OS response. Evaluating disulphide formation is important to understand the effects of OS and the functioning of antioxidant defense systems.[35] Therefore, thiol-disulphide balance is a critical parameter in the evaluation of OS balance.[36]

In conclusion, it is known that paratuberculosis is a worldwide widespread disease affecting a large proportion of dairy cattle herds. If it is not combated with effective control measures, its prevalence and effect will increase gradually. Since it is a disease that causes economic losses by affecting both human and animal health, studies for the prevention, diagnosis, and treatment of the disease are important. In this study, cattle with MAP infection were evaluated in terms of oxidative stress parameters and as a result, it was observed that oxidant-antioxidant balance was disrupted and oxidative stress developed. Therefore, it is thought that oxidative stress markers may be important as biomarkers in the diagnosis and treatment of the disease. In addition, we believe that studies in which the course of the disease can be followed by giving antioxidant preparations to the cattle externally to balance the oxidative stress for therapeutic purposes during the disease process can be tried and this study may be a pioneer in such studies.

Ethics Committee Approval: Ethical approval was obtained from "Ethics Committee Directive" of the Ministry of Agriculture and Forestry Samsun Veterinary Control Institute Animal Experiments Local Ethics Committee. (Date: 03.05.2024, Number:19572899/031-85)

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