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Evaluation of Oxidant/Antioxidant Markers, Cytokines, DNA Damage and Trace Mineral Levels in Goats Naturally Single and Co-Infected with *Theileria ovis* and *Anaplasma ovis*

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ABSTRACT

The impact of co-infections with *Theileria ovis* and *Anaplasma ovis* on caprine health is very limited knowledge and is rarely documented in the literature. Türkiye is endemic for tick-borne diseases (TBDs), posing a risk of co-infections in goats. To evaluate the impact of single and co-infection with *T. ovis* and *A. ovis* on caprine health, four groups of goats were examined: healthy goats, goats infected with *T. ovis*, goats infected with *A. ovis* and goats co-infected with both species. Sera from blood samples was tested for oxidant/antioxidant markers [malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST)], cytokines [interleukins (IL)-6, IL-1, tumor necrosis factor-α (TNF-α)], DNA damage [8-hydroxy-2'-deoxyguanosine (8-OHdG)] and trace minerals [(copper (Cu), zinc (Zn), manganese (Mn), selenium (Se)]. MDA, 8-OHdG, TNF-α, IL-1 and IL-6 levels, CAT and GST enzyme activities were found to be significantly increased in both *Theileria ovis* and *Anaplasma ovis* single- and co-infections ($P<0.05$). In goats, single and co-infected with *A. ovis* showed a statistically significant increase in SOD enzyme activity compared to goats infected only with *T. ovis* ($P<0.05$). In goats co-infected with *A. ovis* and *T. ovis*, considerable decrease in GPx enzyme activity were observed ($P<0.05$). Zn and Se levels in single infected with *A. ovis* indicated a significant decrease. Also, Cu, Zn, and Se levels in co-infected animals decreased significantly ($P<0.05$). Significant increase in lipid peroxidation and antioxidant enzyme activity in goats with single and double infections indicates impaired erythrocyte membrane integrity due to oxidative stress. Moreover, increased proinflammatory cytokine concentrations in all groups indicate that a high degree of systemic inflammation is induced during parasitaemia.

1. INTRODUCTION

Theileria ovis and *Anaplasma ovis* are widespread blood parasites of small ruminants with common geographical distribution in tropical and subtropical regions. Both parasites are tick-borne diseases (TBDs). The causative agents of theileriosis in small ruminants are *Theileria ovis*, *T. lestoquardi*, *T. separata*, which have variable pathogenicity. Small ruminant anaplasmosis is mostly caused by *Anaplasma ovis* and *A. phagocytophilum* (Ahmed et al., 2011; Prajapati et al., 2023). The most common symptoms of anaplasmosis are jaundice and hemolytic anemia. The characteristic clinical symptoms in theileriosis are fever, tachycardia, hemolytic anemia, anorexia, and lymph node enlargement. However, *T. ovis* is known as benign theileriosis and has a mild transient symptom (Ahmed et al., 2011; Prajapati et al., 2023). Although it has been reported that hemolytic anemia may be caused by different reasons, it has been revealed that the underlying mechanism of anemia is erythrocyte oxidative damage. Studies on tick-borne blood parasites (*Theileria* spp., *Babesia* spp. and *Anaplasma* spp.) have shown that reactive oxygen radicals increase in the erythrocytes of infected

ruminants, leading to lipid peroxidation and cell damage (Nazifi et al., 2011; Esmaeilnejad et al., 2012; İlkaya et al., 2020; Uslu et al., 2023). Malondialdehyde (MDA) is the end product of lipid peroxidation in the erythrocyte membrane and is an indicator of the degree of oxidative damage in cells. Lipid peroxidation is usually evaluated by MDA measurement (Özdek et al., 2019). Oxidative damage in erythrocytes also occurs through changes in the activities of antioxidant enzymes involved in the superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) system (De et al., 2012; Jalali et al., 2016). Lipid peroxidation leads to deterioration of erythrocyte morphology and increased osmotic fragility, making erythrocytes susceptible to phagocytosis (Jalali et al., 2016). Increased levels of reactive oxygen radicals in range pathological situations in cells cause oxidative DNA damage. Macromolecular structures are created when oxidative DNA breaks. One of the metabolites that results from oxidative DNA damage is 8-hydroxy-2'-deoxyguanosine (8-OHdG). 8-OHdG is one of the most significant markers of endogenous oxidative damage to DNA. It is created when hydroxyl radicals attack the eighth carbon atom of the guanine nucleotide (Halliwell and Gutteridge, 1999; Ercan and Fidanci, 2012). Activated macrophages are the primary source of proinflammatory cytokines, which play a role in controlling inflammatory reactions. Proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-alpha) act as activators of acute-phase proteins synthesized in the liver. Although proinflammatory cytokines are important in host defense, their unbalanced and excessive production damages the host (Yılmaz and Turgay, 2009; Özdek et al., 2020; Uslu et al., 2023). Assessment of circulating levels of proinflammatory cytokines and antioxidants provides useful information in the evaluation of the severity and clinical outcome of some diseases (Bayram et al., 2020). Certain trace elements, including selenium, copper, and zinc, are crucial parts of the antioxidant defense system. They are used for the synthesis and activity of antioxidant enzymes (Evans and Halliwell, 2001). Copper-zinc SOD (Cu-Zn-SOD) and manganese SOD (Mn-SOD) enzymes in the mitochondria catalyze the conversion of superoxide radicals to hydrogen peroxide (H2O2) and molecular oxygen, the first step in antioxidant defense. Glutathione peroxidase (GPx) contains selenium in its structure. It converts H_2O_2 into water. Therefore, measuring the levels of these trace elements in the serum provides an indication of the status of mineral nutrients. Their coordinated antioxidant role, together with the activity of antioxidant enzymes, is also shown. Decreased levels of these trace elements provide a basis for the formation of oxidative damage (Jalali et al., 2016).

The effects of theileriosis and anaplasmosis infections on oxidative stress markers, antioxidants, and cytokines and trace minerals were evaluated in different hosts. However, to the best of our knowledge, although there are a limited number of studies on anaplasmosis infection of goats, there are no studies on theileriosis infection. Therefore, in this study, serum SOD, CAT, GPx and GST enzyme activities and MDA, 8-OHdG, TNF-α, IL-1, IL-6, Cu, Zn, Mn and Se levels were investigated in goats single and co-infected with *Theileria ovis* and *Anaplasma ovis*.

2. MATERIALS AND METHODS

2.1. Animals and Sampling

This study was carried out with four groups of animals: 10 goats naturally single-infected with *T. ovis*, 10 goats naturally single-infected with *A. ovis*, 10 goats naturally co-infected with *T. ovis* and *A. ovis*, and 10 healthy goats. Blood samples were collected from goats in the Gevas province of Van, Türkiye, between April and June 2022. Blood samples were taken from the vena jugularis of the animals in both anticoagulant-free and anticoagulant (EDTA) tubes. The blood samples were centrifuged at 2000 rpm for 10 min and the serum was separated. Once the serum was transferred into eppendorf tubes, it was kept at -20 °C until it was time for analysis. The Animal Experiments Local Ethics Committee of Van Yuzuncu Yil University provided approval for this research (dated 28/03/2024 and numbered 03-01).

2.2. DNA extraction and polymerase chain reaction (PCR) amplification

Total genomic DNA was isolated from whole blood samples using a commercial DNA extraction kit (Hybrigen Genomic DNA Isolation Kit, Catalog No.: MG-GDNA-01-250, Türkiye), in accordance with the manufacturer's instructions. A final amount of 50–100 µl was used to elute the DNA. After that, DNA extracts were kept at -20°C until needed. *Theileria ovis* infection was screened in all samples with PCR amplification of fragment (520 bp) of the SSU rRNA sequence specific for *T. ovis* by using specific primers TSsr 170F (5'-TCGAGACCTTCGGGT-3') and TSsr 670R (5'-TCCGGACATTGTAAAACAAA-3'), as described by Aktaş et al., (2005). *Anaplasma ovis* infection was screened in all samples with PCR amplification of fragment (852 bp) of the the msp4 sequence specific for *A. ovis* by using specific primers MSP45 (5′-GGGAGCTCCTATGAATTACAGAGAATTGTTTAC-3′) and MSP43 (5′-CCCCGGATCCTTAGCTGAACAGGAATCTTGC-3′), as described by De la Fuente et al., (2007). A master mixture of 20 µl was prepared containing 10 µl of WizPure™ PCR 2X Master mix, 3 µl of template DNA, 1 µl of of each primer (concentration of 10 pmol) and 5 µl of sterile distilled water. The thermal cycling profile was described by Altay et al., (2005) and de la Fuente et al., (2007). Distilled water and DNA extracted from *T. ovis* and *A. ovis* were used as negative and positive controls, respectively. PCR products were electrophoresed in 1.5% agarose gel and sized with 100 bp DNA ladder (Genesta^{TM}).

2.3. Assessment of Biochemical Analysis

SOD (Cat no: SL0106Gt), CAT (Cat no: SL0109Gt), GPx (Cat no: SL0105Gt), and GST (Cat no: SL0108Gt) enzyme activities and MDA (Cat no: SL0104Gt), TNF-α (Cat no: SL008Gt), IL-1 (Cat no: SL0110Gt), IL-6 (Cat no: E002GO), and 8-OHdG (Cat no: SL0107Gt) levels by ELISA (Stat Fax 2100, USA) using commercial test kits (Sun long Biotech Co. , Ltd, China) were measured using. Serum Cu, Zn, Mn and Se levels were measured by ICP-MS (Thermo Scientific).

2.4. Statistical Analysis

Results of the analysis were obtained using the statistical program SPSS (version 20). The normality of the data was assessed for each parameter using the Shapiro-Wilk test. Independent samples t-test was used to determine the difference between the groups. The significance degree between two groups was determined to be $P<0.05$. The results obtained were given as Mean±SD.

3. RESULTS

The PCR assay was used to determine the parasitic infection. *Theileria ovis* and *Anaplasma ovis* infections were confirmed in single and co-infected goats. All the control groups were confirmed as negative for *T. ovis* and *A. ovis*. Significant increases in MDA, 8-OHdG, TNF-α, IL-1, and IL-6 levels and CAT and GST enzyme activities were found in both single-infected and co-infected goats with *T. ovis* and *A. ovis* ($P<0,05$). Significant increase in SOD enzyme activity was found in the single-infected goats and co-infected goats with *Anaplasma ovis* ($P<0.05$). A significant decrease in GPx enzyme activity was found in the co-infected with *T. ovis* and *A. ovis* ($P<0.05$). There was a significant decrease in Zn and Se levels in both goats with *T. ovis* and *A. ovis* and Cu, Zn, and Se levels in co-infected goats with A. ovis ($P<0.05$). The decrease in GPx enzyme activity in both goats with *T. ovis* and *A. ovis* and the increase in SOD enzyme activity in goats with *Theileria ovis* were not statistically significant ($P>0.05$). It was determined that the decrease in Cu, Zn and Se levels in the goats with *T. ovis* and in the Cu level the goats with *A. ovis* was not statistically significant ($P>0.05$). It was determined that the decrease in Mn level did not show a significant difference between healthy and infected animals ($P>0.05$) (Table 1).

4. DISCUSSION

Oxidative stress plays a significant role in the pathophysiology of tick-borne protozoan diseases. MDA (Malondialdehyde) is the most commonly used biomarker to assess the extent of lipid peroxidation in studies related to oxidative stress (Grotto et al., 2009). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) are antioxidant enzymes that protect red blood cells from the damaging effects of free radicals (De et al., 2012; Jalali et al., 2016). The occurrence of oxidative damage can be attributed to changes in the activities of the antioxidant enzymes in question. It has been hypothesized that oxidation of erythrocyte membranes contributes to the development of anemia in goat anaplasmosis (İlkaya et al., 2020). It was observed that there was an increase in lipid peroxidation and a concomitant decrease in antioxidant activity (SOD and CAT) in goats with anaplasmosis as parasitaemia increased (Dhanasree et al., 2019; 2020). It was demonstrated that catalase enzyme activity decreased significantly, and the level of malondialdehyde increased in goats with anaplasmosis (Özdek et al., 2019). Jalali et al., (2016) reported that in goats naturally infected with *A. ovis*, the level of MDA did not change in

correlation with parasitemia, but the activity of SOD enzyme decreased. MDA levels were found to increase in cattle with anaplasmosis, while SOD, CAT, and GPx enzyme activities were found to decrease (El-Ashker et al., 2015; Esmaeilnejad et al., 2018; Değirmençay et al., 2023). In contrast, it has been reported that SOD, CAT, and GPx enzyme activities are significantly increased in sheep infected with *Anaplasma ovis*. Yasini et al., (2014) reported that SOD, CAT, and GPx enzyme activities increased 15 days after infection with *A. ovis* in sheep, although the increase in SOD enzyme activity was not statistically significant. It has been reported that MDA levels, as well as SOD and CAT enzyme activities, increased in cattle infected with *A. marginale* (Abdel Hamid et al., 2014).

In a study conducted on sheep infected with theileriosis, it was documented that malondialdehyde levels increased, superoxide dismutase enzyme activity decreased significantly, while alterations in glutathione peroxidase and catalase enzyme activities were not statistically significant (Baghshani et al., 2011). In another study, it was reported that MDA levels increased, while activities of SOD, CAT, and GPx enzymes decreased significantly at various parasitemia levels (Nazifi et al., 2011). Similarly, it was observed that MDA levels increased, and the activities of SOD, CAT, and GPx enzymes decreased significantly in cattle infected with theileriosis (Razavi et al., 2011; Molayi-Jabdaragi et al., 2020). In a separate study, it was demonstrated that the activities of SOD and GPx enzymes were significantly reduced in the context of theileriosis infection, whereas the CAT enzyme showed an increased activity (Asri Rezaei and Dalir-Naghadeh, 2006). A study conducted on cattle with theileriosis reported a significant increase in MDA levels and GPx enzyme activity. However, the increase in superoxide dismutase enzyme activity was not statistically significant (Neelam et al., 2017).

A review of the literature revealed no studies investigating glutathione S-transferase (GST) enzyme activity in infections caused by *Theileria ovis* and *Anaplasma ovis*. In a study conducted by El-Far et al., (2014), a reduction in GST enzyme activity was observed in patients with babesiosis. The present study demonstrated a statistically significant increase in MDA levels in both the single infection and co-infection with *Theileria ovis* and *Anaplasma ovis*, compared to the healthy goats. The highest increase was observed in the co-infected goats. The data obtained were analyzed in relation to antioxidant enzymes, revealing that superoxide dismutase (SOD) activity increased significantly in all groups except the single-infected group with *T. ovis*. Moreover, catalase (CAT) and glutathione S-transferase (GST) enzyme activities showed significant increases in both the single and co-infected groups with *T. ovis* and *A. ovis* compared to the healthy goats. The increase in these enzyme activities was more prominent in the co-infected goats. A decrease in glutathione peroxidase (GPx) enzyme activity was noted in all infected goats compared to the healthy goats. However, this decrease was statistically significant only in the co-infected goats.

Table 1. Comparison of serum parameters between healthy goats, infected with one parasite, and goats with co-infection.

Parameter	Healty Goats (n=10) (Mean±SD)	Goats with *T. ovis* (n=10) (Mean±SD)	Goats with *A. ovis* (n=10) (Mean±SD)	Goats with co-infection (n=10) (Mean±SD)
MDA (ng/mL)	2,86±0,60	3,99±0,57*	*4,35±0,48	5,02±0,82*
SOD (pg/mL)	23,59±4,95	33,79± 20,64	37,12±19,61*	44,99±15,43*
CAT(ng/mL)	29,08 ±8,99	56,30± 13,85*	63,11±33,02*	71,73±26,14*
GPx (ng/mL)	31,27±2,37	29,32±3,80	29,93±4,02	28,16±2,89*
GST (ng/mL)	6,18±3,79	19,36±7,68*	21,83±10,41*	28,63±12,29*
8-OHdG (pg/mL)	3,55 ±0,59	4,25±0,69*	4,48±1,00*	4,81±0,53*
TNF-α (pg/mL)	23,21 ±4,39	42,84±13,57*	48,05±27,73*	75,88±46,65*
IL-1(pg/mL)	4,86 ±1,35	6,92± 1,39*	8,40 ±1,75*	8,42±2,44*
IL-6 (ng/L)	30,38±1,66	33,16±2,54*	34,48±4,84*	41,13±4,65*
Cu (mg/L)	0,60 ±0,22	0,50 ±0,21	0,44±0,41	0,37±0,19*
Zn (mg/L)	1,55 ±0,25	1,26±0,60	1,27±0,32*	1,12±0,46*
Mn (mg/L)	0,08 ±0,05	0,04±0,05	0,06±0,07	0,07 ±0,07
Se (mg/L)	3,82 ±0,8161	3,49±0,59	3,23±0,27*	3,13 ±0,30*

Statistically significant value = P<0.05. MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, GPx: glutathione peroxidase, GST: glutathione S-transferase, 8-OHdG: 8-hydroxy-2'deoxyguanosine, TNF-α: tumor necrosis factor, IL-1: interleukin-1, IL-6: interleukin-6, Cu: copper, Zn: zinc, Mn: manganese, Se: selenium.

The observed increase in malondialdehyde (MDA) levels in both single-infected and co-infected goats with *Theileria ovis* and *Anaplasma ovis* indicates lipid peroxidation in erythrocyte membranes. The increase in antioxidant enzymes suggests a peak in parasitemia, with the antioxidant capacity being stimulated in response to the emerging stress. In studies of tick-borne protozoan diseases, there is often a reported increase in malondialdehyde (MDA) levels, while findings regarding antioxidant parameters vary. This discrepancy may be attributed to differences in the antioxidant capacity of the animal species studied and the severity of the infection.

8-OHdG is a metabolite produced as a consequence of oxidative DNA damage and serves as a crucial indicator used to quantify this damage (Valavanidis et al. 2009). It has been documented that the level of 8-OHdG is increased in cases of *Plasmodium* and *Trypanosoma* infections (Yano et al., 2008; Herbas et al., 2009; Herbas et al., 2010). Ciftci et al., (2014) showed that the increase in 8-OHdG levels was statistically significant in dogs infected with *Babesia vogeli* compared to healthy goats. The current study found that the level of 8-OHdG was elevated in both the single-infected and co-infected goats with *Theileria ovis* and *Anaplasma ovis* compared to the healthy goats. This increase was statistically significant.

Proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) are crucial in initiating systemic inflammation in response to infection and tissue damage. The acute phase response in parasitic infections is stimulated by proinflammatory cytokines, including IL-1, IL-6 and TNF-α. Interleukin-6 (IL-6) plays a crucial role in the hepatic acute phase response, whereas IL-1 and TNF-α are involved in extrahepatic cases (Murata et al., 2004; Uslu et al., 2023). The current study demonstrated a notable increase in TNF-α, IL-6, and IL-1 levels in both the single-infected and co-infected goats with *Theileria ovis* and *Anaplasma ovis*, compared to the healthy goats. The most substantial increase was observed in the co-infected goats. The results indicate that elevated levels of proinflammatory cytokines are present in both single-infected and co-infected goats, suggesting a significant degree of systemic inflammation induced during parasitemia. In studies conducted on cattle infected with *A. marginale* and sheep infected with *A. ovis*, the levels of TNF-α, IL-6 and IL-1 were found to be significantly increased compared to the healthy goats, which supports our findings (El-Ashker et al., 2015; Uslu et al., 2023). Bayram et al., (2020) demonstrated that the increase in TNF-α and IL-1 levels was statistically significant in sheep with anaplasmosis. It has been demonstrated that macrophages activated by *T. annulata* in cattle secrete increased levels of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6. These cytokines play a crucial role in stimulating both innate and acquired immunity against protozoal agents (Nazifi et al., 2010; El-Sebaei et al., 2014; Aktas et al., 2023). In a study published in 2019, Razmi and colleagues observed a significant increase in IL-6 and TNF-α level in sheep infected with *Theileria*.

The presence of parasitic diseases in animals increases their susceptibility to deficiencies of trace elements and vitamins (Dede et al., 2008; Akış and Dede, 2009; Mis and Oguz, 2022). The changes in trace element concentrations observed in parasitic and infectious diseases are usually the result of a complex mechanism. The impact of these elements on the host immune system depends on the severity and duration of the parasitic infection (Dede et al., 2008; Özdek et al., 2019). In a study conducted on goats with anaplasmosis, Özdek et al., (2019) found a significant increase in Cu levels, along with a decrease in Zn and Mn levels. In another study by Esmaeilnejad et al., (2018), it was observed that Cu, Zn, Mn, and Se levels were decreased in cows infected with *A. marginale* compared to the control group. Similarly, Das et al., (2022) observed a decrease in Cu and Zn levels in cows with anaplasmosis. In contrast to the aforementioned results, Jalali et al., (2016) found no statistically significant decrease in Cu and Zn levels in goats infected with *A. ovis*. In studies conducted on cattle (Razavi et al., 2011) and sheep (Nazifi et al., 2011) with theileriosis, statistically significant decreases in Cu, Zn, Se, and Mn levels were reported. Similarly, studies have indicated that Cu levels are significantly decreased in cattle with theileriosis (Değer et al., 2005; Denizhan et al., 2017). In addition to the findings of the aforementioned studies, it was observed that the levels of trace elements examined in all infected groups were significantly lower than those of the control group. However, the decrease in Zn and Se levels in the single infected group with *A. ovis* and the decrease in Zn, Se, and Cu levels in the co-infected goats were found to be statistically significant. This decrease in trace element levels may be attributed to their utilization as cofactors for antioxidant enzymes such as Cu/Zn-SOD, Mn-SOD, and GPx, or to their insufficient amounts in the diet.

5. CONCLUSION

The significant increase in lipid peroxidation and antioxidant enzyme activity observed in *A. ovis* and *T. ovis* single infected and co-infected goats suggests that the heightened erythrocyte fragility in these groups results from the compromised integrity of erythrocyte membranes due to oxidative stress. The increased concentrations of proinflammatory cytokines observed across all groups suggest a substantial level of systemic inflammation triggered during parasitemia and the most pronounced effects were observed in the co-infected goats.

Authors' declarations

Publication consent

Each author has demonstrated their consent for the publication of the current manuscript.

Data and material availability

All data of this study are provided.

Conflict of interests

All authors have stated the absence of any conflicts of interest.

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Authors' contributions.

L.M: performed the experiments and contributed to the interpretation of the data. B.O: performed the molecular identification of the species by sequence analyses. Y.D. and B.O. wrote and edited the manuscript. The final text was reviewed and approved by all authors.

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