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# Sheep Naturally Infected with *Anaplasma ovis* - Evaluation of Cardiac and Inflammatory Biomarkers

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**ABSTRACT**

***Background*:** Anaplasmosis, also called gall sickness or tropical bovine ehrlichiosis, is an infectious disease caused by species belonging to the genus *Anaplasma* in domestic and wild animals in tropical and subtropical regions. *Anaplasma ovis* and *A. phagocytophilum* are important pathogens of sheep. *A. ovis* is considered the most common species affecting sheep. The infection is usually subclinical and progresses with high fever, anaemia, icterus, weight loss and abortions. This study aimed to investigate changes in cardiac damage markers, oxidative stress and antioxidant status, cytokines, and acute phase proteins in sheep naturally infected with *A. ovis*.

***Materials, Methods & Results*:** For this purpose, a total of 40 animals, including 20 healthy sheep and 20 sheep infected with anaplasmosis, were used. *A. ovis* was diagnosed based on clinical findings and peripheral blood smear. Blood smears were prepared from the ear vein. The smears were stained with Giemsa and examined for the presence of *Anaplasma* spp. Infection was also confirmed by polymerase chain reaction (PCR) analysis. The genomic DNA was isolated from blood, and the MSP-4 gene region was amplified as *A. ovis* specific target gene. Twenty clinically healthy sheep of the same age group, reared under the same conditions and testing negative in the molecular assessment were used as controls. Blood samples were collected from the cephalic vein and and centrifuged to obtain serum. The serum stored at -20°C until the analysis stage. Serum samples were used for the analysis of cardiac damage markers [troponin I (cTnI), creatine kinase MB (CK-MB), lactate dehydrogenase (LDH) and aspartate transaminase (AST)], oxidative stress parameters [malondialdehyde (MDA), total antioxidant status (TAS), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)], cytokines [interleukins IL-6, IL-1β and IL-10, tumour necrosis factor α (TNF-α), and interferon-γ (IFN-γ)] and acute phase proteins [C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin (Hp)]. cTnI and CK-MB levels were measured using a chemiluminescent immunoassay. MDA, TAS, SOD, CAT, GPx, TNF-α, IL-1β, IL-6, IL-10, IFN-γ, SAA and Hp levels were measured by an ELISA reader. LDH, AST and CRP levels were measured in an autoanalyzer. cTnI and LDH levels were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$). The concentration of AST was decreased in infected animals. MDA, TAS, SOD, CAT and GPx levels were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$). The levels of the inflammatory parameters such as TNF-α, IL-1β, IL-10 and IFN-γ were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$). Hp level were significantly increased in the infected group compared with the control group ($P < 0.05$). However, there was no significant change in CK-MB, SAA and CRP concentrations in the infected animals ($P > 0.05$).

***Discussion*:** Ovine anaplasmosis is an obligate intracellular arthropod disease that causes widespread changes in haemato-biochemical, immune response and oxidative stress parameters. Cardiac damage is often overlooked in field conditions due to the lack of adequate knowledge about the pathophysiology of the disease. Our results showed that *A. ovis* infection leads to significant changes in cardiac biomarkers and that the parasite can cause cardiac dysfunction. This is the first report on cardiac damage markers in *Anaplasma*-infected sheep. Additionally, the levels of proinflammatory and oxidative stress markers that may cause functional disorders were also found to be increased. Thus, measuring markers of cardiac function, oxidative stress and inflammation can be a useful tool in the early diagnosis of ovine anaplasmosis.

**Keywords:** anaplasmosis, cardiac damage, acute phase protein, cytokine, oxidative stress, ruminants.

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## INTRODUCTION

Anaplasmosis is an infectious disease caused by species of the genus *Anaplasma* in domestic and wild animals in tropical regions [24]. The infection is usually subclinical and progresses with high fever, anaemia, icterus and abortions [42]. Malondialdehyde (MDA) is the most commonly measured biological lipid peroxidation product that reflects oxidative stress in tissues [22]. Troponin I (cTnI), creatine kinase MB (CK-MB), aspartate transaminase (AST) and lactate dehydrogenase (LDH) levels are used as markers of cardiac damage [19,29]. Acute phase protein (APP) are mainly proteins synthesized in the liver, and their concentrations vary as a result of inflammatory stimulation [11]. In ruminants, unlike in other species, the most important APP is haptoglobin (Hp) [34,38]. Some proinflammatory cytokines such as interleukin (IL) and tumour necrosis factor (TNF) are responsible for inducing the release of APP [6]. IL-6 is more effective in hepatic acute phase response. Interferon-γ (IFN-γ) has a direct effect on the production of APP [3].

Since anemia and secondary hypoxia occur in piroplasmosis [18,20,43], we hypothesized that anaplasmosis causes cardiac dysfunction in sheep. A previous study on cardiac damage markers in *Anaplasma* infection was conducted with cattle [44] but to our knowledge, there is no report on parameters of anaplasmosis in sheep. Thus, this study aimed to investigate cardiac damage markers, oxidative stress and the antioxidant status, and changes in cytokines as well as acute phase proteins in sheep naturally infected with *A. ovis*. This study also discusses the role of cardiac damage in the consequences of the severe pathogenic effects of *A. ovis*.

## MATERIALS AND METHODS

### *Animals and samples*

This study was conducted with 20 sheep naturally infected with *Anaplasma ovis* in the Konya province of Turkey in 2019. *A. ovis* was diagnosed based on clinical findings and blood smear. Infection was also confirmed by polymerase chain reaction (PCR) analysis. According to laboratory findings, no other blood parasites were present in the sheep. In addition, microscopic examination with different stains such as Gram's stain and acid-fast staining was performed to confirm infection with *A. ovis* only and exclude other causes of anemia. Twenty clinically healthy sheep of the same age group, reared under the same conditions, and testing negative in the molecular assessments were used as the control group.

Blood samples were collected from the cephalic vein, placed in gel tubes and centrifuged (2,000 *g*, 15 min) to obtain serum. The serum was poured into eppendorf tubes and stored at -20°C until the analysis stage. The animals were not treated for the disease prior to sampling.

### *Preparation of blood smear and microscopic examination*

Thin blood smears of both groups were prepared from the ear vein, air-dried, and fixed with methanol for 1 min. The smears were stained with Giemsa stain for 30 min, washed under tap water and examined for the presence of *Anaplasma* spp. inside the erythrocytes in 100 oil-immersion fields. More than 20 microscope fields were examined to determine the level of parasitemia.

### *Molecular analysis*

Genomic DNA was extracted from the blood samples using a commercial DNA blood kit[1] for DNA purification according to the manufacturer's instructions. Briefly, after DNA extraction, PCR was performed using the following specific primer pair for *A. ovis*: MSP-4-F (5′-TGAAGGGAGCGGGGTCATGGG-3′) and MSP-4R (5′ GAGTAATTGCAGCCAGGCACTCT-3′). The PCR reaction mixture had a final volume of 25 µL, containing 5 µL of 5X Master Mix[2] 0.1 µL of 0.2 µM of each primer, 3 µL of the DNA template and 16.8 µL of MQ water. The thermal profiles of the PCR were completed according to the specified protocol of Torino *et al*. [47]. Previously sequence-confirmed DNA templates were used as positive controls, and MQ water served as the negative control.

### *Measurement of cardiac biomarkers*

Cardiac biomarkers such as cTnI and CK-MB in the serum were measured using a chemiluminescent immunoassay (Immulite 1000)[3]. The serum concentrations of LDH and AST were measured in an autoanalyzer (ILab-300)[4].

### *Measurement of oxidative stress*

MDA (MDA-586 Kit)[5], total antioxidant status (TAS) [Total Antioxidant Status Kit][6], SOD (Superoxidase Dismutase Assay Kit)[7], CAT (Catalase

Assay Kit)[7] and GPx (Cayman Glutathione Peroxidase Assay Kit)[7] in the serum were measured by an ELISA reader (MWGt Lambda Scan 200)[8] according to the manufacturer's instructions.

*Measurement of cytokines and APP*

TNF-α (Sheep TNFα ELISA kit)[9], IL-1β (Sheep IL-1β ELISA kit)[9], IL-6 (Sheep IL-6 ELISA kit)[9], IL-10 (Sheep IL-10 ELISA kit)[9], IFN-γ (Sheep IFN-γ ELISA kit)[9], SAA (Sheep SAA ELISA kit)[9] and haptoglobin (Sheep Hpt/HP ELISA kit)[9] in the serum were measured by an ELISA reader (MWGt Lambda Scan 200)[8] according to the kit procedure. CRP concentration was measured in an autoanalyzer (ILab-300)[4].

*Statistical analysis*

Research data were presented as mean±SE and tested for normality. Normally distributed values were evaluated with the two-sample t-test and other parameters with the Mann-Whitney U test (SPSS 22.0). A value of $P < 0.05$ was accepted as statisticallly significant.

## RESULTS

The concentrations of cardiac biomarkers of both controls and naturally infected sheep are presented in Table 1. cTnI and LDH levels were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$), but no change in CK-MB values was observed ($P > 0.05$). The concentration of AST was decreased in diseased sheep ($P < 0.05$).

Table 2 shows the levels of oxidative stress parameters in the healthy (control) and infected sheep. MDA, TAS, SOD, CAT and GPx levels were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$).

Table 3 shows the levels of the inflammatory parameters in the healthy (control) and infected sheep. The concentrations of TNF-α, IL-1β, IL-10 and IFN-γ were significantly increased in the infected animals compared to the healthy ones ($P < 0.05$), while there was no significant change in IL-6 concentration ($P > 0.05$).

The concentrations of APP in both controls and naturally infected sheep are presented in Table 4. According to our data, a significant increase was observed in the Hp level ($P < 0.05$), while there was no significant change in SAA and CRP concentrations ($P > 0.05$). Among the acute phase proteins, Hp was the most sensitive parameter that changed in diseased sheep. Therefore, the increase in Hp concentration suggests that it may be a sensitive indicator for the detection of acute infection and inflammation in sheep naturally infected with *A. ovis* ($P < 0.05$).

**Table 1.** Serum cardiac biomarkers of healthy sheep and those of naturally infected with *Anaplasma ovis* (mean ± SE).

| Biomarkers | Healthy sheep (Control) (n = 20) | Infected sheep (n = 20) | *P* -value |
|---|---|---|---|
| cTnI (ng/mL) | 0.03 ± 0.01 | 0.13 ± 0.07 | < 0.001 |
| CK-MB (ng/mL) | 0.14 ± 0.01 | 0.15 ± 0.01 | > 0.769 |
| LDH (U/L) | 206.65 ± 11.64 | 364.45 ± 16.76 | < 0.001 |
| AST (U/L) | 110.45 ± 2.76 | 74.25 ± 3.45 | < 0.001 |

n: number; cTnI: cardiac Troponin I; CK-MB: creatine kinase; LDH: lactate dehydrogenase; AST: aspartate aminotransferase.

**Table 2.** Serum oxidative status parameters of healthy sheep and those of naturally infected with *Anaplasma ovis* (mean ± SE).

| Biomarkers | Healthy sheep (Control) (n = 20) | Infected sheep (n = 20) | *P*-value |
|---|---|---|---|
| MDA (U/mL) | 10.91 ± 0.68 | 18.07 ± 2.19 | < 0.0028 |
| TAS (mmol/L) | 58.18 ± 5.21 | 107.78 ± 15.52 | < 0.0036 |
| SOD (U/mL) | 0.83 ± 0.17 | 6.28 ± 1.72 | < 0.0005 |
| CAT(nmol/min/mL) | 10.77 ± 2.66 | 26.76 ± 4.46 | < 0.0001 |
| GPX(nmol/min/mL) | 26.79 ± 2.38 | 60.31 ± 10.84 | < 0.0003 |

n: number; MDA: malondialdehyde; TAS: total antioxidant status; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase.

**Table 3.** Cytokines of healthy sheep and those of naturally infected with *Anaplasma ovis* (mean ± SE).

| Biomarkers | Healthy sheep (Control) (n = 20) | Infected sheep (n = 20) | *P*-value |
|---|---|---|---|
| TNF-α (pg/mL) | 35.71 ± 3.01 | 77.56 ± 12.32 | < 0.0002 |
| IL-1β (pg/mL) | 21.40 ± 1.94 | 44.37 ± 4.51 | < 0.0001 |
| IL-6 (pg/mL) | 95.32 ± 7.60 | 153.25 ± 22.40 | > 0.0531 |
| IL-10 (pg/mL) | 111.19 ± 5.00 | 180.30 ± 21.19 | < 0.0016 |
| INF-γ (pg/mL) | 85.03 ± 5.90 | 146.90 ± 24.42 | < 0.0337 |

n: number; TNF-α: tumour necrosis factor; IL-1β: Interleukin -1 beta; IL-6: Interleukin-6; IL-10: Interleukin-10; INF-γ: Interferon gamma.

**Table 4.** Acute phase proteins of healthy sheep and those of naturally infected with *Anaplasma ovis* (mean ± SE).

| Biomarkers | Healthy sheep (Control) (n = 20) | Infected sheep (n = 20) | *P*-value |
|---|---|---|---|
| Hp (mg/mL) | 106.90 ± 5.79 | 216.05 ± 24.93 | < 0.0001 |
| SAA (ng/dL) | 16.75 ± 0.12 | 16.70 ± 0.10 | > 0.884 |
| CRP (mg/dL) | 0.64 ± 0.01 | 0.65 ± 0.01 | > 0.477 |

n: number; Hp: haptoglobin; SAA: serum amyloid A; CRP: C-reactive protein.

## DISCUSSION

Ovine anaplasmosis is an obligate intracellular arthropod disease that causes widespread changes in haemato-biochemical, immune response and oxidative stress parameters [25,26,45]. However, studies have not focused on assessing cardiac biomarkers. Cardiac damage is often overlooked in field conditions due to the lack of adequate knowledge about the pathophysiology of the disease. Anemic hypoxic state and acid-base and electrolyte disturbances can lead to the disruption of myocardial cell membrane integrity, resulting in cardiac damage that clinically manifests as a change in cardiac biomarkers [18,20]. In experimental ovine babesiosis, general tissue haemorrhage has been observed, with the degeneration and necrosis of cardiac muscle fibres [21]. There is strong evidence that canine babesiosis [31], equine babesiosis [10], bovine babesiosis [39], and bovine [18,20] and ovine theileriosis [43] cause heart damage. However, little is known about cardiac dysfunction in ovine anaplasmosis. Changes in cardiac biomarkers indicate cardiovascular involvement in anaplasmosis pathophysiology.

cTnI and CK-MB are primary markers used in the diagnosis of acute myocardial damage [19]. Previously, we measured these markers and noted that they could also be used successfully to detect heart damage in sheep [12]. In this study, an increase cTnI level was detected in sheep naturally infected with *A. ovis* (Table 1; $P < 0.05$). cTnI is unique to cardiac tissue, making it a very sensitive serum biomarker of physical or metabolic myocardial damage compared to CK-MB [20,30]. Recent studies have reported that cTnI levels are increased in bovine babesiosis, equine piroplasmosis and ovine babesiosis [2,17,27,39]. Similarly, increased serum cTnI levels in dairy cows naturally infected with Anaplasma [44]. However, there seems to be no report of cTnI changes in ovine anaplasmosis. According to our results, an increase in cTnI level can be accepted as an indicator of myocardial damage. Our data represent the first study to evaluate cardiac damage markers in ovine anaplasmosis. In the present study, there was no significant change in CK-MB activity in sheep anaplasmosis. Similar findings regarding CK-MB in infections of blood protozoa such as ovine theileriosis [4]. However, contrary to our findings, previous study found a significant increase in serum CK-MB activity in bovine anaplasmosis [44].

LDH and AST levels are also classic markers of cardiotoxicity [46]. LDH is an enzyme found in erythrocytes, and its level increases after hemolysis. In this study, an increase in LDH level was detected due to the anemia observed in sheep naturally infected sheep with Anaplasma (Table 1; $P < 0.05$). Some studies have reported similar patterns of LDH and AST in bovine babesiosis and ovine theileriosis [4, 39]. Muscle and liver diseases may result in an increase CK-MB,

AST and LDH activity, but the overall increase in the serum concentration of cTnI and increase in the activity of other enzymes (LDH) in this study may indicate myocardial damage during the pathogenesis of ovine anaplasmosis.

Significant changes in oxidative stress parameters were observed in sheep naturally infected with Anaplasma in this study. MDA is an essential biological parameter in revealing lipid peroxidation [14]. In this study, the levels of MDA and antioxidant enzyme activity, including CAT, TAS, GPX and SOD, significantly increased in the infected group compared to the control group (Table 2; $P < 0.05$). According to this study's results, the increased MDA level reflects lipid peroxidation, and oxidative damage occurs in erythrocytes during infection. Some studies have suggested that oxidative damage in erythrocytes is associated with anaemia. Since the erythrocyte membrane is susceptible to lipid peroxidation, the number of free radicals that cause lipid peroxidation in erythrocytes increases in the presence of blood protozoa such as *Theileria* spp. and *Babesia* spp., and cell damage occurs due to this increase [28]. Increased MDA levels have been reported in *Anapalasma ovis* infection in goats [37] and *A. marginale* infection in cattle [13,16], and the present findings are in agreement with these reports. Unlike this result, the MDA level did not change in goats in naturally infected with *A. ovis* [25]. Previous studies have shown that *A. marginale* increases erythrocyte osmotic fragility in experimental and natural infections in cattle, associated with morphological changes in erythrocytes [35,40].

SOD, CAT and GPx are enzymes found in RBCs to counter the toxic effects of reactive oxygen species (ROS) [33] and protect erythrocytes from lipid peroxidation effects [5,7]. In this study, antioxidant enzyme activity, including CAT, TAS, GPX and SOD, significantly increased in the infected group compared to the control group (Table 2; $P < 0.05$). High MDA levels are generally observed in hemoparasitic diseases, but in many studies, different results have been reported regarding antioxidant enzyme activity. Increased activity of SOD and CAT has been reported in *A. marginale* infection in cattle [1,13], *A. ovis* infection in sheep [45] and *Babesia gibsoni*-infected dogs [5], and the present findings are in agreement with these reports. In a recent study, SOD, CAT and GPx activity was reported to increase in acute infection in sheep experimentally infected with *A. ovis*, but Gpx activity in erythrocytes decreased with the progression of the disease [45]. On the other hand, one study reported that SOD and TAC levels were significantly lower in *A. marginale*-infected cattle [9]. Another study also found decreased activity of CAT in goats with anaplasmosis [37]. SOD, TAS and GPx levels have been reported to decrease in horses infected with *A. phagocytophilum* [32]. According to our study results, increased serum CAT, TAS, GPx and SOD levels in sheep infected with *A. ovis* may be associated with continued antioxidant enzyme production during the acute phase of infection. It has been suggested that the peak of parasitemia coincides with the highest levels of SOD and CAT enzyme activity in sheep experimentally infected with anaplasmosis [45].

Proinflammatory cytokines play an important role in initiating systemic inflammation against infection and tissue damage. The acute phase response is stimulated by some proinflammatory cytokines such as IL-1, IL-6 and TNF-α due to tissue damage in parasitic infections. In this study, TNF-α, IL-1β, IL-10 and INF-γ levels increased in the group infected with *A. ovis* (Table 3; $P < 0.05$). Similar findings also have been reported in cattle infected with *A. marginale* [13] and horses infected with *A. phagocytophilum* [32]. The concentrations of circulatory TNF-α and IFN-γ were clearly increased in cattle infected with *A. marginale* [35]. According to our results, increased concentrations of proinflammatory cytokines in all infected animals may indicate a high degree of induction of systemic inflammation during parasitemia. In addition, the high level of IL-10 may indicate that the inflammation is suppressed, and the level of INF-γ may indicate that the synthesis of immunoglobulin is stimulated.

It is known that Hp is a major APP in inflammatory diseases of ruminants [15,41]. Other study reported that the levels of major APP increased severalfold from basal levels as a result of inflammation or trauma[ 34]. Although C-reactive protein (CRP) is not considered an acute phase protein for cattle, its concentration increases in inflammation and infections [38]. In cattle, serum amyloid A (SAA) is raised more in cases of acute inflammation [23]. In this study, no change was observed in SAA and CRP levels in animals naturally infected with Anaplasma ($P > 0.05$), whereas the Hp level increased (Table 4; $P < 0.05$). One study reported a significant increase in Hp concentration in the early and chronic stages of ovine anaplasmosis which is

agreement with our findings [26]. Increased Hp levels have also been reported in bovine anaplasmosis [8,36]. This significant increase shows that inflammatory responses are induced in sheep infected with *A. ovis*. Hp can also be a useful tool in the diagnosis and prognosis of anaplasmosis in sheep.

## CONCLUSION

In conclusion, our results showed that *Anaplasma ovis* infection leads to significant changes in cardiac biomarkers and that the parasite can cause cardiac dysfunction. A significant increase in cTnI level was observed and could serve as a biomarker for detecting cardiac damage in *A. ovis* infection. Additionally, the levels of proinflammatory and oxidative stress markers that may cause functional disorders were determined to increase. The significant increase in antioxidant enzyme activity and lipid peroxidation of erythrocytes indicates the result of increased erythrocyte fragility due to damage to the erythrocyte membrane by oxidative stress. Measuring markers of cardiac function, oxidative stress and inflammation can be a useful tool in the early diagnosis of ovine anaplasmosis. Further studies are needed to uncover the mechanisms underlying cardiac complications and better understand the pathogenesis of this parasitic disease.

MANUFACTURERS

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***Ethical approval.*** The sheep owners approval was obtained for sampling. All procedures used in this study was approved by the Ethical Committee of the Faculty of Veterinary Medicine University of Selcuk, Turkey (No. 2019/68).

***Declaration of interest.*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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