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Investigation of Some Markers of Inflammation in Sheep with Anaplasmosis*

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Abstract: Anaplasmosis is an arthropod-borne parasitic disease that infects blood cells and frequently seen in sheep. This study was planned to evaluate inflammation marker levels in sheep infected with anaplasmosis. In this research, the blood samples used were obtained from 20 healthy and 20 Anaplasmosis sheep brought to Van Metropolitan Municipality Slaughterhouse. Determination of control and patient groups, clinical symptoms of the disease, Giemsa stained blood smears and serological method (cELISA) were used. Serum samples of 91 sheep collected from *Anaplasma* spp. When examined serologically for the presence of antibodies of the species, 73.6% (67/91) were found to be seropositive in terms of Anaplasmosis. The blood of the sheep with high ELISA inhibition values and showing Anaplasma morulae form in the peripheral blood smears, were used for analysis. Granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) levels in 20 sheep whose positivity was detected were identified with the Elisa kit. IL-1β (ng/L), TNF-α (ng/L), GM-CSF (ng/L) levels from among the pro-inflammation parameters were found to increase. It is thought that researching the mechanisms related to inflammation developing against Anaplasma will contribute to studies for diagnosis and prevention of Anaplasma infections for future immunotherapy research.

Keywords: Anaplasmosis, GM-CSF, Interleukin-1β, Interleukin-6, TNF-alpha.

Anaplazmozisli Koyunlarda Bazı Inflamasyon Markırlarının Araştırılması

Öz: Anaplazmozis koyunlarda sıklıkla görülen, kan hücrelerini enfekte eden artropod kaynaklı ve ekonomik kayıplara neden olabilen paraziter bir hastalıktır. Bu çalışma Anaplazmozis ile enfekte koyunlarda bazı serum inflamasyon markır seviyelerini değerlendirmek için planlanmıştır. Yapılan araştırma için kullanılacak olan kan örnekleri, Van Büyükşehir Belediyesi Mezbahanesi'ne getirilen 20 sağlıklı ve 20 Anaplazmozis'li koyundan sağlandı. Kontrol ve hasta gruplarının belirlenmesi, hastalığın klinik belirtileri, giemsa boyalı kan frotileri ve serolojik yöntem (cELISA) yardımıyla yapıldı. Toplanan 91 koyunların ait serum örneği *Anaplazma spp.* türlerinin antikorlarının varlığı yönünde serolojik olarak incelendiğinde %73.6'sı (67/91) Anaplazmozis yönünden seropozitif bulundu. Kan frotisinde Anaplazma etkenleri görünen ve ELISA inhibisyon değeri yüksek çıkan koyunların kanları analizler için kullanıldı. Anaplazmozis'li koyunlar teşhis edildikten sonra pozitif ve negatif olarak gruplandırıldı. Pozitifliği tespit edilen 20 koyun serumunda granülosit makrofaj koloni uyarıcı faktör (GM-CSF), interlöykin-1β (IL-1β), interlöykin-6 (IL-6), tumor nekroz faktör- alfa (TNF-α) düzeyleri elisa kiti ile kit prosedürüne göre belirlendi. Proinflamasyon parametrelerinden serum IL-1β (ng/L), TNF-α (ng/L), GM-CSF (ng/L) düzeylerinin negatif gruba göre istatiksel olarak yükseldiği tespit edildi. Anaplazmaya karşı gelişen inflamasyon ile ilgili mekanizmaların incelenmesi, ileride geliştirilecek immunoterapi araştırmaları için Anaplazma enfeksiyonlarının teşhis ve önlemeye yönelik çalışmalara katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Anaplazmozis, GM-CSF, İnterlökin-1β, İnterlökin-6, TNF-alfa.

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INTRODUCTION

Anaplasma species are tick-borne intracellular rickettsial pathogens that harm human and animal health. The major species that cause anaplasmosis in sheep are *Anaplasma ovis* and *Anaplasma phagocytophilum.* Among predisposing factors are air temperature, ticks with dense intestinal parasites, and poor health conditions (1). Anaplasmosis is usually subclinical. Symptoms such as weight loss, abort, and decrease in milk and meat yield are observed in infected animals (2). Significant economic losses can occur in sheep infected with *Anaplasma* spp.

Pro-inflammatory cytokines are mainly produced by activated macrophages and play a role in the regulation of inflammatory reactions. Interleukin (IL)-1β, IL-6, and tumor necros factor(TNF-α), which are pro-inflammatory cytokines, play a significant role during the pathological pain process (3) In various studies, it has been suggested that evaluating pro-inflammatory cytokine and antioxidants levels in circulation might be useful in understanding the severity and clinical results of some diseases (4-6).

The present study aimed to contribute to diagnosis-treatment options and immunotherapy practices related to *Anaplasma* by discussing immune system regulation mechanisms. It also aimed to contribute to take necessary measures and develop types of treatment by determining the levels of systemic pro-inflammation factors, which are the preparative of many degenerative diseases.

MATERIALS and METHODS

This research was approved by the local ethics committee of Yuzuncu Yil University (YUHADEK-Approval No: 2019/01).

The material of this study was the blood samples drawn from 91 sheep brought to the Van Municipality slaughterhouse. Giemsa-stained blood smears were examined under the microscope. Anaplasma was identified on the basis of its morphology (2). The serum samples collected from 91 sheep were serologically examined in terms of the presence of antibodies of *Anaplasma* spp. and 73.6% (67/91) of them were found to be seropositive in terms of anaplasmosis. The blood samples of the sheep whose *Anaplasma* agents had been observed in the blood smears and ELISA inhibition values had been high were used for the analyses. Control and patient groups were determined using the clinical symptoms of the disease, blood smears with Giemsa stain, and serological method (cELISA) (*Anaplasma* antibody test kit, c-ELISA, no: 282- 2VMRD-USA). After the sheep with Anaplasmosis were diagnosed, they were grouped as positive (20) and negative (20).

Tumor necrosis factor alpha (TNF-α) (catalog no: YLA0031SH), Interleukin-6 (IL-6) (catalog no: YLA0060SH), Interleukin-1beta (IL-1β) (catalog no: YLA0053SH), Granulocyte macrophage colony stimulating factor (GM-CSF) (catalog no: YLA0123SH) levels in the sheep serums were measured with a commercial ELISA kit. The standard curve of the measurements was drawn and the results were evaluated.

Statistical Analysis

SPSS software was used for the statistical analysis of the obtained data. First of all, distribution normality test was performed to assess whether the groups showed normal distribution. The groups were found to show abnormal distribution since significance value of Shapiro-Wilk test was below statistical significance level of 0.05. Therefore, the statistical differences between the groups were evaluated using Mann Whitney U test. It was used in the comparison of the serum IL-6, TNF-α, GM-CSF, and IL-1β levels of the control and the experimental groups. Parameters with a P-value lower than 0.05 were considered significant.

RESULTS

Intergroup statistical evaluation of the serum IL-1β (ng/L), TNF-α (ng/L), IL-6 (ng/L), and GM-CSF (ng/L) levels were determined (Table 1).

Table 1. For all groups-1β (ng / L), TNF-α (ng / ml), IL-6 (ng / L), GM-CSF (ng / ml) mean values and standard deviations (X ± SX).
Tablo 1. Tüm gruplara ait IL-1β (ng/L), TNF-α (ng/ml), IL-6 (ng/L), GM-CSF (ng/ml) değerleri ortalamaları ve standart sapmaları (X±SX).

Parameters	Control (NK) (n=20) $X \pm S_X$	Anaplasma positive (PK) (n=20) $X \pm S_X$
IL-1 (ng/L)	0,8297±0,19805[a]	0,9587±0,13702[b]
IL-6 (ng/L)	3,14910± 08414	3,1733±0,03860
TNF-α (ng/L)	1,2104±0,25144[a]	1,4913±0,33207[b]
GM-CSF (ng/L)	1,4691±0,23632[a]	1,7087±0,19920[b]

[a,b]Comparison of some blood parametric means found in each application group (For the relevant blood parameter, the statistical difference between the group means with different letters in the same row is important (P<0.05).

Statistically, IL-1β values of the positive (anaplasma +) group were found to be higher than those of the negative (healthy) group (P<0.05). No statistically significant difference was found in the serum IL-6 levels of the sheep with anaplasmosis and the ones in the control group. TNF-α levels rose in the group with anaplasmosis (P<0.05). GM-CSF levels of the positive sheep were found to be statistically higher than those of the negative group (P<0.05).

DISCUSSION and CONCLUSION

As a result of tissue damage occurred in parasitic or other infections, an acute phase response (APR) with a non-specific reaction is activated. The acute phase response is activated via some pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β). Production of some plasma proteins increases in this period. These proteins, whose synthesis is increased by stimulation, prevent tissues from getting more damage and accelerate healing in this way.

However, unwanted effects occur when also the APR lasts longer than it should. Cytokines are the main mediators effective in these metabolic changes (7). Haptoglobin (Hp) and serum amyloid-A (SAA), main acute phase proteins (APP) in ruminants, have been emphasized in many studies as clinically helpful parameters under several conditions. These parameters have been observed to increase in theileriosis in cattle (8). Again, it was reported that these parameters increased more than 10 times in Hp levels in ruminants infected with *Anaplasma* when class with in good health cattle. Hp and SAA concentrations may be good indicators of inflammation with parasitemia associated with *A. marginale* in cattle. Inflammation after the *Anaplasma* infection stimulates APP synthesis. For this reason, evaluation of acute phase response in cattle infected with *A. marginale* is important for determining the inflammation (9). Peracute anaplasmosis, which has a high mortality rate, is characterized by several hours of clinical symptoms that most frequently develop in purebred animals and dairy cows with high yield (10). The concentrations of pro-inflammatory cytokines are correlated with the prognosis of sepsis and multiple organ dysfunction syndromes (11). Various studies have argued that it might be useful to evaluate the pro-inflammatory cytokine levels in circulation in understanding the severity of some diseases and predicting the clinical outcomes (5,6).

TNF has a significant role in regulating the host immune system (12). It was reported that TNFα is released rapidly in various infections, has different impacts on metabolism but is usually the main regulator of the pro-inflammatory cytokine response (13). Studies have reported that TNF-α stimulates macrophages and increase the amount of nitric oxide (NO) and reactive oxygen species (ROS) in this way. Some other studies have reported that during 25 acute inflammations the parasites of the active macrophages neutralize parasites by phagocytosis and producing toxic substances such as superoxide, NO, peroxynitrite (14,15). IL-6 and TNF-α play a role in the creation of clinical symptoms in acute diseases and in immune system against parasitic infections (16). IL-1β, synthesized by the defense system, activates the IL-6 release in living things (17). TNF-α

rose in the infection with the experimental *T. annulata* agent in ruminants (18). In the current study, similarly, TNF-α and IL-1β levels were found to increase.

IL-6 is a mediator that has important roles in inflammation and causes to synthesize amyloid-A and C-reactive proteins. At the same time, it also enables T cells to grow and differentiate (19). It is highly important for the living things that the relationship between anti-inflammation and inflammation be balanced in infections. IL-10, with an anti-inflammatory effect that is released by macrophages and granulocytes and prevents cytokine production, presses for the production of pro-inflammatory cytokines (IL-6, TNFα, IL-1β,) (20). IL-6 level was found to increase in *Anaplasma* disease caused by *Anaplasma phagocytophilum.* The IL-6 level was reported to rise in another study on cattle. The reason for this increase may be explained with the increase in IL-6 level after anaplasmosis agents activate macrophages (21). In also this study, it rose numerically but it was not statistically significant. This might be because IL-10 now starts suppressing.

In a study on cattle with anaplasmosis, the reason for the increase in the lipid peroxidation, IL-6, NO, and heat protein levels was the stimulation of host immune system by the *Anaplasma*-agent parasites and as oxidative stress emerged together with the inflammation, it was reported that detection of Heat shock protein 27 (HSP27), malondialdehyde (MDA), IL-6, and NO levels would be useful in diagnosis of anaplasmosis (21,22).

IL-6 is effective in naturally acquired immunity as well as activates Interleukin-10 (IL-10) synthesis in T lymphocytes. IL-10 inhibits the cell-mediated immune response. Cytokines primarily take part in the defense system for infections (23). IL-10, whose synthesis increases as a result of IL-6 stimulating T lymphocytes, suppresses IL-1 TNF- α, and IL-6 production. It creates this effect by suppressing the production of cytokines like IL-6, TNF-α, and IL-1 at transcriptional and post-transcriptional levels. As a result, IL-10 suppresses pre-inflammation cytokines and the acute phase responses of the hosts, and in this way, helps reduce the severity of the inflammation. Interleukins play a significant role in immunity in parasitic infections, but their unstable and much synthesis may be harmful. These harmful effects are damage in tissues, lipid peroxidation, weight loss, and shock (24).

The neutrophils infected with *A. phagocytophilum* are activated for the production of chemokines (IL-8) and cytokines (IL-6) (25), biologically active compounds with many effects including changes increased in vascular permeability. IL-6 also contributes to parenchyma damage and both IL-6 and IL-10 can affect as compensatory neuroprotective factors (26).

The granulocyte-macrophage colony-stimulating factor (GM-CSF), which is the hematopoietic growth factor, is the cytokine that stimulates the proliferation and differentiation of blood cells. Hematopoietic growth factors are commercially used for infectious diseases in clinics. It was shown in some studies that it reduced post-chemotherapy neutropenia in acute neutropenia cases after cancer chemotherapy in patients with sarcoma, lymphoma, and solid tumor. GM-CSF may cause fever response depending on an increase in IL-1 that can be controlled with paracetamol. In peripheral blood stem cell transplantations, peripheral blood cells stimulated by G-CSF and GM-CSF are collected by leukapheresis and applied in bone marrow aplasia that develops as a result of cytotoxic treatment. Neutropenic and thrombocytopenic duration shortened and antibiotic use time decreased in these patients. This practice did not change the relapse and mortality rates (27). It was shown that GM-CSF increased neutrophil levels in non-severe cases of aplastic anemia. Although the number of reticulocytes increased, the transfusion requirement of the patients remained unchanged. In severe aplasia, the response is much lower. In myeloid leukemia and myelodysplastic syndromes, GM-CSF can create a positive effect by activating leukemic cells and causing the

chemotherapeutic agent to increase its effectiveness on these cells in this way. In HIV infections, GM-CSF has been reported to correct some neutrophil function defects by increasing neutrophil, eosinophil and monocyte levels (27,28).

One of the most important effects of GM-CSF is its success in the treatment of invasive candida and fusarium infections developed after bone marrow transplants and leukemia treatment. A partial improvement was observed in aspergillus infections (28). Its effect on parasites in vitro has also been shown in cell culture models of *Trypanosoma cruzi* and *Leishmania tropica* (27). However, the effect of GM-CSF on parasitic infections should be investigated at the clinical level.

In the present study, GM-CSF values were found to be high in anaplasmosis. Its high presence was evaluated as the response of the immune system to correct the effect of anaplasmosis factor on blood cells. It may be useful to use its commercial form as a supplement to alleviate the disease symptoms.

In conclusion, our findings in *Anaplasma* infection clearly show that high levels of the measured inflammatory markers and the extent of the responses serve as an important prognostic indicator. The parasitemia levels increased the most with IL-1 and therefore it can serve as a biomarker for inflammation. The measured inflammatory markers associated with *Anaplasma* infection may be a useful tool to prescribe the outcome of the disease. It is thought that the increase in knowledge about the mechanisms of the immune response in anaplasmosis and the factors that affect it will significantly contribute to the knowledge required for future immunotherapy research and the vaccine studies against anaplasmosis.

Conflict of interest

The authors declare that they have no conflict of interest.

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