Investigating Erythrocyte Membrane Lipid and Protein Oxidation with Na^+/K^+ATPase Activity in Caprine Anaplasmosis

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SUMMARY

Anaplasmosis is an infectious disease that is caused by the genus Anaplasma belonging to the family Anaplasmataceae in the order Rickettsiales and is seen in the mammals inhabiting tropical and subtropical climate zones. *Anaplasma ovis* and *A. phagocytophilum* are the most remarkable species causing anaplasmosis in goats. Anaplasmosis induces both cellular and humoral immunity. As animals develop a long-term immunity against the disease, it becomes difficult to treat the disease. Immunity develops mainly depending on premunition. Increased osmotic fragility of erythrocytes in animals infected with anaplasmosis has been found, and it has been reported that this may be developed by various immune-mediated mechanisms including oxidative damage. Moreover, it may also be associated with high cell membrane ATPase activity and erythrocyte morphological changes. The aim of this study was to evaluate the effect of anaplasmosis on erythrocyte membrane malondialdehyde (MDA), advanced oxidation protein products (AOPP), Sodium-potassium adenosine 5'-triphosphatase (Na^+/K^+ATPase), and hematological and biochemical parameters in goats. For this purpose, 45 male hair goats (35 infected and 10 healthy) that were brought to the slaughterhouse of Van Metropolitan Municipality were enrolled in the study. In infected goats, the hematological and biochemical concentrations of RBC, Hb, Hct, MCV, MCH, MCHC as well as serum total protein, albumin, total cholesterol and TIBC were found significantly lower, however concentrations of of WBC, globulin, total bilirubin, direct bilirubin, triglyceride, and iron as well as enzyme activities of AST, ALT, GGT were found higher when compared to the healthy goats ($p<0.05$). In addition, the MDA and AOPP levels were markedly increased in erythrocyte membrane from infected animals while the Na^+/K^+ATPase enzyme activity was significantly decreased compared to the the healthy goats ($p<0.05$).Therefore, it can be concluded that oxidative stress in erythrocyte membrane may play an important role in the pathogenesis of anemia in caprine anaplasmosis.

KEY WORDS

AOPP, Caprine Anaplasmosis, Goat, MDA, Na^+/K^+ATPase.

INTRODUCTION

Anaplasmosis is an infectious disease that is caused by Anaplasma species and leads to significant losses in meat, fleece and milk production in tropical and subtropical climate zones with inadequate feeding conditions. Anaplasma species are obligate intracellular rickettsial pathogens that have a size of 0.3-1 micron and no cytoplasm and are localized in the erythrocytes of hosts and close to their membrane[1]. Ovine and caprine anaplasmosis are generally associated with *Anaplasma ovis* and *A. phagocytophilum* and this disease is transmitted biologically by various tick species (*Boophilus spp., Rhipicephalus spp., Hyalomma spp., Ixodes spp.* and *Dermacentor spp.*) and mechanically by some diptera species (*Tabanid spp., Stomoxys spp.* and *Melophagus ovinus*)[2].

The clinical symptoms of the disease vary based on the general condition, breed, and age of the infected animal. In sheep and goats, acute anaplasmosis starts with general depression, numbness, and fever of 40-41°C and develops with weight loss, progressing anemia, dehydration, and hepatitis along with a rapid decrease in milk yield[3]. Anaplasmosis induces both cellular and humoral immunity. As animals develop a long-term immunity against the disease, it becomes difficult to treat the disease. Immunity develops mainly depending on premunition[4]. It has been reported that the osmotic fragility of erythrocytes increases in the animals infected with anaplasmosis and this may be developed by several immune-mediated mechanisms including oxidative damage[5] and also may be associated with high cell membrane ATPase activity and erythrocyte morphological changes[6].

A great number of studies have focused on the effect of reactive oxygen species in the pathogenesis of parasitic infections and their cellular defense mechanisms. Some studies have revealed the effects of anaplasmosis on oxidative markers and antioxidants [5,7]; however, there has been no report on erythrocyte membrane lipid peroxidation product malondialdehyde (MDA), advanced oxidation protein products (AOPP) and sodi-

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um-potassium adenosine 5'-triphosphatase (Na^+/K^+ATPase) in the goats infected by Anaplasma species. This study was conducted in order to reveal the effect of anaplasmosis on erythrocyte membrane MDA, AOPP, and Na^+/K^+ATPase as well as hematological and biochemical parameters in infected goats.

MATERIALS AND METHODS

A total of 35 male hair goats diagnosed with anaplasmosis upon clinical and laboratory findings, and 10 healthy male hair goats, all of which had an average age of between 1.5 and 2.0 years and were brought to the slaughterhouse of Van Metropolitan Municipality, were used as the study material.

Thin blood smear was prepared using the blood sample obtained from ear tip of each goat, thereafter these smears were stained using Giemsa method and examined using a light microscope at x100 magnification in terms of anaplasmic forms. Also, blood samples were taken from jugular vein of the animals into EDTA tubes and into tubes without anticoagulant agent. The blood samples taken into the tubes without anticoagulant agent were centrifuged at 840 g for 10 minutes in order to obtain serum samples. Commercial c-ELISA kit (Anaplasma antibody test kit, c-ELISA, no: 282- 2VMRD-USA) was used in these serum samples in order to determine the anaplasma antibodies.

The determinations of serum total protein, albumin, globulin, total bilirubin, direct bilirubin, triglyceride, cholesterol, total iron binding capacity (TIBC) and iron levels as well as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT) enzyme activities were performed using a commercial kit in a modular auto analyzer (Roche, Germany). In the blood samples taken into EDTA tubes, red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell (WBC) parameters were determined in the blood count device (Vet. Wasson MC- 1200). Then, these blood samples were centrifuged at 4000xg$^+$4°C for 5 minutes, plasma were separated, and the remaining erythrocyte pellet was used for extraction of erythrocyte membrane[8]. In the erythrocyte membrane obtained, MDA, AOPP, Na^+/K^+ATPase and protein analyses were performed. MDA determination in erythrocyte membrane was performed based on Ohkawa et al.,[9] method. This method is based on formation of a pink-colored chromogen by MDA, a lipid peroxidation product, which reacts when heated with thiobarbituric acid in acidic environment. The absorbance of this colored complex at 532 nm is directly proportional to MDA concentration. For AOPP measurement, the method used by Witko-Sarsat et al.[10] was applied by making some revisions. This method is based on the spectrophotometric measurement of AOPP in the acidic environment in the presence of potassium iodide that allows to show maximum absorption at 340 nm. In membrane suspension, Na^+/K^+ATPase activity measurement was performed using the commercial kit that works based on biotin double antibody sandwich technique (Goat Na^+/K^+ATPase, Catalog No: YLA0092GO). The protein content of erythrocyte membrane was determined by Lowry method, in which bovine serum albumin is used as standard[11].

Statistical analysis

The data was statistically analyzed using SPSS 22 program. Statistical differences between the groups were evaluated using Mann-Whitney "U" test. The obtained results were given as X±SE. The value of $p<0.05$ was accepted as statistically significant.

RESULTS

When the hematological and biochemical parameters were compared, it was determined that the RBC, Hb, Hct, MCV, MCH, MCHC, total protein, albumin, and total cholesterol levels of the infected group decreased and their WBC, globulin, total bilirubin, direct bilirubin, triglyceride, iron, TIBC levels and AST, ALT, and GGT enzyme activities increased compared to the values of the healthy group ($p<0.05$). When the parameters in erythrocyte membrane were examined, it was determined that the erythrocyte membrane MDA and AOPP levels of the infected group increased and their Na^+/K^+ATPase enzyme activity decreased compared to the values of the healthy group ($p<0.05$).

DISCUSSION

The most important species leading to ovine and caprine anaplasmosis in the eastern Turkey are *A. ovis* and *A. phagocytophilum*. The disease is diagnosed by using microscopic examination and serological or molecular methods[12]. In anaplasmosis infections, changes are observed in the blood parameters of the host in relation with the duration and severity of disease. Hematologic disorders such as anemia, thrombocytopenia, and leukopenia are frequently observed in animals infected with anaplasmosis [13,14]. Although the certain mechanism of anemia is not clear, the infected erythrocytes become hemolyzed. This is associated with the direct damage of lipid peroxidation, increasing during the course of the disease, to membrane structure as well as the weak antioxidant defense.[6,15]Present study revealed a significant decrease in RBC, Hct, and Hb values in the goats with anaplasmosis[15,16,17,18,19]. The decrease of these parameters may be resulting from intravascularly hemolyzed erythrocytes, damaged due to pyrogens released by the Anaplasma species, and failure to remove these erythrocytes from circulation by reticuloendothelial system (RES)[16,18]. Additionally, some studies reported no significant difference in these parameters in the *A. ovis*-infected goats[5,20].

It has been stated that in the anaplasma infection, the decrease in hemoglobin and erythrocyte indices generally causes macrocytic hypochromic[15,16], normochromic normocytic[5] and macrocytic normochromic[17] anemia in small ruminants. In the present study, MCH, MCHC, and MCV values, accompanying the change of hematocrit values in the infected goats decreased significantly, which may indicate that microcytic hypochromic anemia developed in these animals. It was determined in this study that there was a significant increase in WBC count of the infected animals[5,18,20]. This increase may be associated with the activation of lymphoid organs and chronic antigenic stimulation depending on the presence of anaplasma[21]. On the other hand, Ahmadi-hamedani et al.,[16] determined that WBC count decreased. The blood parameters such as urea, creatinine, cholesterol, triglyceride, albumin, globulin, total pro-

tein, total lipid, and bilirubin and also the important enzymes such as amylase, AST, ALT, ALP, and CK are crucial parameters for determining biochemical profile. Based on these parameters, important information is obtained on the pathogenesis and prognosis of the disease[22,23]. Parasites cause significant changes in the biochemical profile of their hosts [16]. In the previous studies, it was determined that anaplasmosis caused an increase in AST, GGT[24], ALT, and ALP[25] enzyme activities in infected animals. Differently from the foregoing results, it was determined that AST activity decreased[15] and ALT activity did not change[26]. In this study, ALT, AST and GGT enzyme activities, which are specific for liver, significantly increased in the infected goats. Hypoxia developing in anaplasmosis due to anemia may increase activity of these enzymes by causing liver damage [27]. The measurement of bilirubin caused by the decomposition of erythrocytes is used in the diagnosis and treatment of the liver, hemolytic, hematologic, and metabolic disorders[22]. In the present study, it was determined that bilirubin concentrations significantly increased in infected goats[15,24]. This significant increase in bilirubin levels may be associated with the hemolysis of the parasitic erythrocytes and hepatic function disorder[15]. Differently from these results, it has been reported in the studies that total bilirubin level decreases[26] or remains within the physiological limits[25].

Many diseases affect the concentration, distribution or synthesis rates, degradation or excretion of serum proteins albumin, and globulin synthesized by liver. The changes in total protein value, fractional distribution and A/G ratio are generally used to assess the disorders of the protein metabolism[22,23]. Previous studies reported that serum total protein and globulin levels significantly increased in the animals infected with anaplasmosis[15,26]. However, other studies indicated that the increase in the total protein[24,25] and albumin[15,24] levels was not significant in infected animals. On the other hand, Kumar et al.,[26] found that the serum albumin levels of the infected animals decreased. In this study, it was determined that while the serum total protein and albumin levels of the infected goats decreased, their globulin levels increased. The decreased albumin level may be due to hepatic impairment or due to the fact that it is an acute phase protein. On the other hand, the increase in serum globulin level may be associated with increased γ-globulin as a response to antigenic stimulation. The blood lipid profile of ruminants changes based on ration, age, gender, pregnancy, genetics, season, lactation, and liver and gallbladder diseases[28]. The serum cholesterol and triglyceride levels in anaplasma infection reveal different results. While Ahmadi-hamedani et al.,[24] determined that there was no significant difference between healthy and infected goats in terms of cholesterol and triglyceride levels, Khaki et al.,[15]found a significant decrease in their cholesterol level. The results of this study showed that the triglyceride level significantly increased but the cholesterol level significantly decreased in the infected goats. High triglyceride level may be associated with the adipose tissue lipolysis that stimulates its production in the liver. And the low total cholesterol level may be due to the fact that its normal synthesis interrupts based on liver damage. Iron is an essential element for many living creatures and has a vital role. 60-70% of iron in the organism is included in the hemoglobin in erythrocytes. TIBC is a measure of the transferrin concentration in blood[29]. It has been determined that the serum iron level increases significantly in the sheep infected with anaplasmosis due to anemia and the decrease in TIBC level is not significant[15]. On the other hand, Jalali et al.,[5] found that the increase in the iron level was not significant in the goats infected with *A. ovis*. It was

Table 1 - Hematological and biochemical parameters in healthy and anaplasmosis goats.

	Parameters	Healthy group (n = 10) X ± SE)	Infected group (n = 35) (X ± SE)
	WBC (m/mm^{-3})	8.69±0.41	11.93±0.80*
	RBC (M/mm^{-3})	15.31±0.63	6.41±0.21*
	Hb (g/dl^{-1})	9.79±0.43	5.56±0.30*
	Hct (%)	29.39±1.24	14.24±0.69*
Hematological	MCV (fl)	20.14±0.79	11.77±0.33*
	MCH (pg)	5.96±0.2	4.23±0.18*
	MCHC (g/dl^{-1})	35.10±1.08	27.82±0.81*
	AST (U/L^{-1})	100.85±9.57	189.42±18.68*
	ALT (U/L^{-1})	20.00±1.07	37.00±4.78*
	GGT (U/L^{-1})	40.71±0.81	96.14±16.02*
	Albumin (g/L^{-1})	29.57±1.34	24.71±0.75*
	Globulin(g/dL^{-1})	43.14±2.26	63.42±4.91*
	T protein (g/L^{-1})	92.14±5.78	69.14±2.52*
Biyochemical	T bilirubin (mg/dL^{-1})	0.66±0.03	0.92±0.03*
	D bilirubin (mg/dL^{-1})	0.36±0.01	0.53±0.02*
	T cholesterol (mgvdL^{-1})	91.86±7.69	52.37±1.95*
	Triglyceride (mg/dL^{-1})	16.43±1.89	31.71±6.74*
	Iron (µg/dL^{-1})	108.28±7.08	141.00±8.95*
	TIBC (µg/dL^{-1})	149.14±16.21	254.71±25.51*

Compared to healty group: *p<0.05. WBC: Leukocyte, RBC: Erythrocyte, Hb: Haemoglobin, Hct: Haematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, GGT: Gamma glutamyltransferase, TIBC: Total iron-binding capacity, T: Total, D: Direct

Table 2 - Erythrocyte membrane MDA and AOPP levels with Na^+/K^+ATPase enzyme activity in healthy and anaplasmosis goats.

Parameters	Healthy group (n = 10) (X ± SE)	Infected group (n = 35) (X ± SE)
MDA (nmol/g^{-1} protein)	4.01±0.19	5.91±0.23*
AOPP (nmol/g^{-1} protein)	0.41±0.01	0.54±0.02*
Na^+/K^+ ATPase (nmol/mg^{-1} protein)	3.33±0.18	2.97±0.16*

Compared to healty group: *p<0.05. MDA: Malondialdehit, AOPP: Advanced oxidation protein products, Na^+/K^+ ATPase: Sodium-potassium adenosine 5'-triphosphatase

determined in the present study that the serum iron level significantly increased and TIBC level significantly decreased in the infected goats. The increase in the iron level is probably due to the iron releasing from intravascular hemolysis of erythrocytes. The decreased TIBC level may be associated with the decrease of transferrin, which is a negative acute phase reactant, due to the inflammation developing as a result of disease. In the host cells infected by different parasite species, the amount of reactive oxygen species (ROS) increases and therefore, cell and tissue damages occur. ROS induces the oxidation of polyunsaturated fatty acids in biological systems and leads to the formation of lipid peroxidation products. MDA is a lipid peroxidation product. The analysis of MDA levels is used to measure the lipid peroxidation level and free radical levels. As erythrocyte membrane is exposed to continuous high oxygen concentration which is rich in polyunsaturated fatty acids, it is highly sensitive to lipid peroxidation[30]. No data has been found on the MDA level of erythrocyte membrane in the goats infected with anaplasmosis. However, it was determined that MDA level increased in the plasma of the cattle infected with anaplasmosis[30,31] and it did not change in the serum of sheep[6]. It was found in this study that anaplasma infection elevated the MDA level of erythrocyte membrane in goats. This increase may be associated with the lipid peroxidation induced based on the increase of ROS generating during the infection. Also, increasing number of iron ions might have aggravated the oxidative damage, as a strong oxidative catalyst forming free radicals. The oxidation of proteins as a result of the covalent modification directly with ROS or indirectly with the secondary products of oxidative stress has a role in the etiology or progress of several disorders and diseases. AOPP, included in the protein oxidation products, is used in determining the oxidation level[32]. Upon the literature review, no data was found on the AOPP levels of erythrocyte membrane in goats injected with anaplasmosis. However, it was determined that serum AOPP level increased in malaria[33,34] and babesiosis[35]. It was found in this study that anaplasma infection elevated the AOPP level of erythrocyte membrane. This increase may be due to the fact that it caused protein oxidation characterized by the changes in the structure and functions of ROS in erythrocyte membrane. Na^+/K^+ATPase is a membrane-associated transport enzyme responsible for maintaining the membrane integrity and the ion balance in cell. Na^+/K^+ATPase, directly or indirectly, controls numerous basic cellular functions and the regulation of this enzyme is effective in the etiology of various pathological processes[36]. Also, it takes place on the top among the enzymes that are affected by the formation of ROS that damages cells[37]. Although there has been no study evaluating the Na^+/K^+ATPase activity of erythrocyte membrane in the infections caused by Anaplasma species, it has been determined that Na^+/K^+ATPase activity decreases in the infections caused by malaria species[37,38,39] and *Babesia ovis*[39]. Although the mechanisms underlying the inhibition of Na^+/K^+ pump are unclear, Staines et al.,[38] reported that many changes occurred in the physical and chemical characteristics of erythrocyte after malaria infection, and any of them may change the activity of the endogenous transport systems. In addition, it was asserted that new permeability pathways (NPPs) induced by parasites may affect the activity of the endogenous carriers[39]. In this study, it was determined that the Na^+/K^+ATPase activity of erythrocyte membrane decreased in goats infected with anaplasmosis. The Na^+/K^+ATPase activity loss considered to develop due to the above reasons may accelerate the hemolysis of erythrocytes by leading to the disruption of intracellular ionic balance.

CONCLUSIONS

The results have revealed that there are significant changes in hematological and biochemical parameters and anemia and oxidative stress are common complications of anaplasmosis in ruminants. In addition, this study showed an increase in MDA and AOPP levels and a decrease in Na^+/K^+ATPase activity in the erythrocyte membrane in goats infected with anaplasmosis. Therefore, it can be concluded that oxidative stress in erythrocyte membrane may play an important role in the pathogenesis of anemia in goats infected with anaplasmosis.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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