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Evaluation of Acute Phase Response and Oxidative Damage in Sheep Naturally Infected with *Streptococcus Pluranimalium* using Haptoglobin, Nitric Oxide, and Malondialdehyde Levels*

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The purpose of this study was to evaluate of acute phase response and oxidative damage in sheep naturally infected with *Streptococcus pluranimalium* using haptoglobin (Hp), nitric oxide (NO), and malondialdehyde (MDA) levels. The animal material of the study consisted of 60 sheep infected with *S. pluranimalium* (Infected group) aged between 4-6 years and 15 healthy sheep (Control group) aged 4-6 years old. Skin scrapings samples were obtained from infected sheep for microbiological analyses. Blood samples were taken from *vena jugularis* of animals in to vacuum tubes for biochemical analysis. From the samples taken from infected sheep, *S. pluranimalium* was identified by using VITEK 2 Compact Bacterial Identification and Monitoring System. Serum Hp and NO levels were determined by the spectrophotometric method and MDA levels were determined by the thiobarbituric acid (TBA) reaction. Hp (0.17±0.01 g/L), ($P<0.001$) MDA (1.67±0.04 μmol/L) and NO (31.14±1.61 μmol/L) ($P<0.01$) levels of the infected group were found to be significantly higher than the control group. It was concluded that high serum MDA and NO levels indicate that oxidative damage takes place in naturally infected sheep with *S. pluranimalium.* However, it can be assumed that higher Hp and low Alb concentrations might be an indicator of acute-phase response in the infected animals.

Key Words: *Acute phase response, oxidative damage, sheep, Streptococcus pluranimalium*

Streptococcus Pluranimalium ile Doğal Enfekte Koyunlarda Akut Faz Yanıt ve Oksidatif Hasarın Haptoglobin, Nitrik Oksit ve Malondialdehit Düzeyleri Kullanılarak Değerlendirilmesi

Bu çalışmanın amacı *Streptococcus pluranimalium* ile doğal enfekte koyunlarda haptoglobin (Hp), nitrik oksit (NO) ve malondialdehit (MDA) düzeylerini kullanarak akut faz yanıtı ve oksidatif hasarı değerlendirmektir. Çalışmanın hayvan materyalini 4-6 yaş arasında olan *S. pluranimalium* ile enfekte 60 adet koyun (Enfekte grup) ve 4-6 yaş arasında olan 15 adet sağlıklı koyun (Kontrol grubu) oluşturmuştur. Mikrobiyolojik analizler için enfekte koyunlardan deri kazıntısı ve biyokimyasal analizler için hayvanların vena jugularislerinden kan örnekleri alındı. Enfekte koyunlardan alınan örneklerden *S. pluranimalium,* VITEK 2 Kompakt Bakteri Tanımlama ve İzleme Sistemi kullanılarak tanımlandı. Serum Hp ve NO düzeyleri spektrofotometrik yöntemle, MDA düzeyleri ise tiyobarbitürik asit (TBA) reaksiyonu ile belirlendi. Enfekte grubun Hp (0.17±0.01 g/L), ($P<0.001$) MDA (1.67±0.04 μmol/L) ve NO (31.14±1.61 μmol/L) ($P<0.01$) düzeyleri kontrol grubundan anlamlı olarak daha yüksek bulundu. Sonuç olarak, yüksek serum MDA ve NO düzeylerinin *S. pluranimalium* ile doğal enfekte koyunlarda oksidatif hasarın meydana geldiğini gösterdiği sonucuna varıldı. Bununla birlikte, daha yüksek Hp ve düşük Alb konsantrasyonlarının, enfekte hayvanlarda akut faz yanıtının bir göstergesi olabileceği varsayılabilir.

Anahtar Kelimeler*: Akut faz yanıt, oksidatif hasar, koyun, Streptococcus pluranimalium*

Introduction

Streptococcus pluranimalium was first described as a new species of the *Streptococcus genus* in 1999 (1). *S. pluranimalium* is promiscuous, in terms of its host and tissue tropism since it has been isolated from various tissues of multiple domestic animals and humans (1). *S. pluranimalium* has been reported to cause subclinical mastitis in dairy cows (1, 2), and many bovine reproductive diseases (abortion, stillbirth, metritis vaginitis, and vulvitis) (2, 3). Additionally, this bacterium has been isolated from human patients with subdural empyema, infective endocarditis, and brain abscess (4-6). On the other hand; pathogenic mechanisms of *S. pluranimalium* are actually unknown at the present time. However, there are insufficient reported data on *S. pluranimalium* infection in sheep.

Reactive oxygen species (ROS) are produced during physiological and metabolic processes and can lead to detrimental oxidative reactions under conditions of excessive production (7, 8). Oxidative damage may develop during situations such as protozoal (9, 10), viral (11, 12) and bacterial infections (13). The primary targets of ROS are

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polyunsaturated fatty acids (PUFAs) in cell membranes. The resulting lipid peroxidation (LPO) may lead to the damage of the cell structure and functions (14, 15). In addition, the decomposition of lipid hydroperoxides yields a wide variety of end-products, including malondialdehyde (MDA). One of the most frequently used ROS biomarkers, providing an indication of the overall LPO intensity is MDA (13-16).

Nitric oxide (NO) is a signalling molecule that plays a key role in the pathogenesis of inflammation (17). Its release is stimulated by macrophages activated by cytokines (18). In conclusion, large amounts of NO are synthesized, exceeding the physiological NO production (17, 18). NO is a substantial molecule included in physiological and pathological processes in animals (17). The activity of NO in cellular defense mechanisms includes participation in tissue injury and the mediation of inflammatory processes and apoptosis (18). However, NO is stated to play an important role in the primary defense against several species of bacteria (19, 20), viruses (11, 21, 22) and parasites (23).

Acute-phase response (APR) mostly occurs during infection and inflammation. The aim of these reactions is to isolate and destroy the infectious agents to prevent ongoing tissue damage, and to restore homeostasis. One of the main features of APR is the hepatic production of acute-phase proteins (APPs). The blood concentration of APPs generally increases within 8h of stimulation, reaches the maximum level in 24-48h (24). APPs may be used for the differentiation of bacterial and viral infections, for the differential diagnosis of clinical, subclinical, acute and chronic diseases (24, 25). The most important major APPs for the determination of sheep diseases are haptoglobin (Hp) (24-26). Hp is APP binding free haemoglobin in the blood. Hp concentrations are increased during acute infection but decreased with treatment or chronicity (24, 25). Nevertheless, their concentration remains high in chronic cases if stimulation continues (25). However, no study was encountered in sheep on the related infection, and no adequate studies have been found on oxidative damage and acute phase response. Therefore, in this study, it was aimed to evaluate the acute phase response and oxidative damage in sheep naturally infected with *S.pluranimalium* using Hp, NO, and MDA levels.

Material and Methods

Research and Publication Ethics: On a farm located in Erzurum with 150 Morkaraman sheep aged 4-6 years old, the owner applied us for diagnosis and treatment as some sheep had pyrexia, anorexia, excessive weakening, necrotic ulcerative dermatitis in the tail area, loss of appetite and weight loss. The flock of sheep was examined after obtaining the “informed consent form” from the owner. The animal material of this study consisted of 60 symptomatic sheep (infected group) and 15 healthy sheep (control group).

Animals: The animal material of the study consisted of infected sheep from a 150-animal sheep herd (Morkaraman breed) in Erzurum province and healthy sheep from the same herd. The infected group consisted of 60 infected sheep aged 4-6 years (Morkaraman breed); while the control group consisted of 15 healthy sheep (Morkaraman breed) aged 4-6 years old. This study was conducted in accordance with ethical rules.

Blood and Skin Scrapings Sampling: Blood samples were taken from the *jugular veins* into vacuum tubes without anticoagulant (Vacutainer, BD-Plymouth, UK) for serum analyses. The blood samples were centrifuged at 3000 *g* for 10 min at room temperature. The sera were separated and stored at –80°C until analysed. Skin scrapings samples were obtained from sick sheep with suspected infection for microbiological analysis.

Bacteriological Analysis: From the samples taken from infected sheep, *S. pluranimalium* was isolated and identified by using VITEK 2 Compact Bacterial Identification and Monitoring System (Biomérieux, Inc., Hazelwood, MO, USA).

Biochemical Analyses

Malondialdehyde (MDA) and Nitric Oxide (NO) analyses: Serum MDA levels were measured by the thiobarbituric acid (TBA) reaction according to the method described by Yoshioka et al. (27). The end products were read at 535 nm. Serum NO levels were determined according to the method described by Miranda et al. (28). Nitrate is reduced to nitrite by $VaCl_3$, and then in an acidic environment nitrite was reacted with sulphanilamide to produce coloured diazonium compound, which was read at 540 nm.

Haptoglobin (Hp) and Albumin (Alb) Analyses: Serum Hp concentrations were determined spectrophotometrically according to the method which have been previously reported by Skinner et al. (29). Serum Alb concentrations also were measured spectrophotometrically (Epoch, Biotek, USA) using a commercial test kit (Biolabo, France).

Statistical Analysis: The SPSS software program (Version 20.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The distribution of the data between the groups was evaluated using the Kolmogorov-Smirnov test. Levene’s test was used to test whether variances were homogenous. Parametrically distributed groups were compared using the t-test (Independent-Samples t-test). The correlation among parameters was determined by the Pearson Correlation test. All data were presented as the mean and standard error of the mean (Mean±SEM). The results were assessed at a 95% confidence interval and a significance level of $P < 0.05$ (30).

Results

Pyrexia, anorexia, excessive weakening, necrotic ulcerative dermatitis in the tail area, death within 5 to 10 days were important clinical findings in infected sheep (Figure 1).

Serum levels of Hp, Alb, MDA and NO in healthy and infected sheep are given in Table 1. Hp (0.17±0.01 g/L) (P<0.001), MDA (1.67±0.04 μmol/L) and NO (31.14±1.61 μmol/L) (P<0.01) levels of the infected group were found significantly higher than the control group. The serum Alb concentrations in the infected group were not statistically different from the control group (P>0.05). Correlations between parameters of the infected group were presented in Table 2. While there was a positive correlation between Hp, NO, and MDA levels of the infected group, there was a significant positive correlation between Hp concentrations and MDA levels (P<0.05). However, a negative correlation was determined between NO, MDA levels, and Alb concentrations.

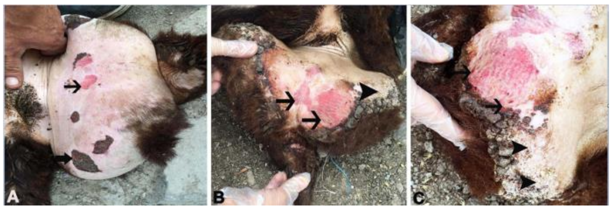

Figure 1. Necrotic-ulcerative dermatitis in the ventral of the tail, ranging from 1-3 cm to 10-15 cm in size. **(A):** Primary effect (bold arrow), **(B-C):** Progressing lesions, ulcerations (thin arrow), necrotic mass (arrow heads).

Table 1. Hp, Alb, MDA and NO levels in healthy and infected sheep

Parameters	Healthy (n=15) Mean±SEM	Infected (n=60) Mean±SEM	P
Hp (g/L)	0.11±0.01	0.17±0.01	<0.001
Alb (g/dL)	3.18±0.12	2.92±0.06	>0.05
MDA (µmol/L)	1.35±0.10	1.67±0.04	<0.01
NO (µmol/L)	22.07±3.70	31.14±1.61	<0.01

Hp: haptoglobin; Alb: albumin; MDA: malondialdehyde; NO: nitric oxide

Table 2. Correlation between parameters in infected sheep

Parameters	Hp	NO	MDA	Alb
Hp	1.000	0.198	0.243*	0.012
NO		1.000	0.129	-0.141
MDA			1.000	-0.174
Alb				1.000

Hp: haptoglobin; NO: nitric oxide; MDA: malondialdehyde; Alb: albümin; *:P<0.05

Discussion

There have been insufficient studies of *S. pluranimalium* infection in sheep worldwide. Therefore, this study aimed to evaluate the acute-phase response and oxidative status in infected sheep. While this bacterium causes problems with endocarditis and brain abscess in humans (4-6), it is reported that it causes mastitis, abortion, and stillbirths in cattle (2, 3), meningoventriculitis in a calf (31), respiratory disease in dogs (32), and septicaemia and endocarditis in broiler chickens (33). On the other hand, in only one report, Foster et al. (34) isolated the agent from two sheep abortion materials, especially from stomach and liver samples of the fetus. At the same time, as clinical findings, it was reported that the sheep were pyrexic at the time of the abortion. In the presented study, pyrexia, anorexia, excessive weakening, weight loss, necrotic ulcerative dermatitis in the tail area (Figure 1), and death within 5 to 10 days were important clinical findings in the infected sheep.

Common causes of oxidative stress are infections, inflammation, and toxaemias (35). Infections and inflammations activate inflammatory cells that play important roles in the host's defense (15). Since major inflammatory cells, such as neutrophils and/or macrophages, generate a variety of ROS and release various proteases, tissue damage and destruction occur (15, 35). In addition, one of the many biological targets of oxidative stress is lipids. PUFAs, in particular, are the frequently targeted class of biomolecules. ROS-induced oxidation of PUFAs in biological systems results in the formation of LPO products (15, 35). MDA is a breakdown product that is quantified as a measure of lipid hydroperoxides, and is accepted as an indicator of elevated oxidative stress in the body (36, 37). In sheep, oxidant status has been identified in metabolic (38, 39), parasitic (9, 10, 40), and viral diseases (11, 12), as well as in physiological conditions, such as age (41) and pregnancy (42). In the present study, serum MDA levels were significantly increased in infected sheep compared to healthy sheep (P<0.01). In this case, it has been consistent with the previous reports. These increases can be considered as an indicator of excessive free radical production in infected sheep.

NO is a prominent molecule in defense against many microorganisms (17, 18). NO, which is produced by macrophages in bacterial infections, shows antibacterial properties against bacteria and host defense depends on the concentration of NO (18). It was reported that NO concentrations in animals with bacterial (19, 20), viral (11, 21, 22) and parasitic diseases (23) increased compared to healthy controls. In addition, it has been determined that NO levels increase in bacterial diseases such as traumatic reticuloperitonitis (43) and traumatic pericarditis (44). Similarly, in the present study, NO concentrations were high in infected sheep as in the above reports. This increase was thought due to inducing of NO synthesis by macrophages defending the organism against infection.

Haptoglobin belongs to a group of transporter plasma proteins that bind the free haemoglobin (45). This defines its bacteriostatic effect as well as antioxidant activity (26, 45) Many studies have demonstrated the importance of Hp as a clinically helpful parameter for measuring the occurrence and severity of inflammatory responses in sheep with various infectious diseases (12, 46-52).

Pepin et al. (46) investigated the changes in Hp concentrations in lambs experimentally infected by *Corynebacterium pseudotuberculosis*. They reported the rapidly increasing Hp concentration to peak in plasma after subcutaneous inoculation. In a similar study, Eckersall et al. (47) explored the differences in plasma levels of Hp during experimental ovine caseous lymphadenitis induced by *C. pseudotuberculosis.* The results showed significantly raised Hp concentrations on day 7 after inoculation and these values were statistically significant until the 15th day. In another study, Bastos et al. (49) also stated that there was no significant difference between serum Hp concentrations in seropositive and seronegative sheep during caseous lymphadenitis in Santa-Ines sheep. However, later in 11 sheep that have not developed peripheral abscesses, a significantly higher Hp concentration was observed. Fasulkov et al. (50) investigated plasma Hp concentrations during experimentally induced *Staphylococcos aureus* mastitis in goats and showed an increase of the Hp as early as the 8th hour with the most significant differences from baseline values by the 24th and 48th h after infection. On the other hand, Chalmeh et al. (51) found a rapid elevation in Hp levels during experimentally induced endotoxaemia in sheep by lipopolysaccharide from *Escherichia coli.* In the present study, Hp concentrations were significantly higher in infected sheep (Hp; 0.17±0.01g/L versus 0.11±0.01g/L) compared to the healthy control group ($P<0.001$). These values were observed to be similar to different studies conducted in previous years (45-49). Therefore, it can be said that Hp concentrations are important in the pathogenesis and monitoring of *S. pluranimalium* infection. In addition, there was a significantly positive correlation between Hp and MDA levels of the infected sheep ($P<0.05$). These findings may indicate that APR and oxidative damage occurs simultaneously in infected sheep and triggers each other.

Albumin is the major negative APP. During the APR the demand for amino acids for synthesis of the positive APPs is markedly increased, which necessitates reprioritization of hepatic protein synthesis. Therefore, Alb synthesis is down-regulated and amino acids are shunted into the synthesis of positive APPs (53, 54). In addition, it has been stated that the serum concentrations of Alb, are affected by impaired liver function, reduced intestinal absorption, and starvation (24, 55). In the presented study, the serum Alb concentrations in infected sheep were not statistically different from the control group. However, the decrease in Alb concentration may be related to the synthesis of APPs and this finding may indicate that hepatic Alb synthesis was affected by APR.

In conclusion, high serum MDA and NO levels indicate that oxidative stress takes place in naturally infected sheep with *S. pluranimalium.* Higher Hp and low Alb concentrations in the infected animals compared to the healthy ones is also an indicator of APR in these animals.

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