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RESEARCH ARTICLE

Oxidative Stress and Total Sialic Acid Levels in Sheep Naturally Infected with Pox Virus

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A B S T R A C T

This study was performed to determine the levels of serum nitric oxide (NO), malondialdehyde (MDA), total sialic acid (TSA), and total oxidant capacity (TOC) and total antioxidant capacity (TAC) as well as histopathological changes in sheep naturally infected with pox virus. The study material comprised of 40 Sheep pox infected and 20 non-infected Akkaraman mixed sheep aging between 1 and 2.5 months. Diagnosis of the disease was based on the clinical and histopathological observations. Blood samples were collected from the infected and healthy animals, and serums were separated. The levels of NO (36.65±1.10 nmol/ml), MDA (5.87±0.14 µmol/L), TSA (88.51±0.82 mg/dl) as well as TOC (685.05±10.84 µmolH_2O_2Eqv/L) and TAC (1.01±0.03 mmol Trolox Eqv/L) were determined in infected animals and compared to the levels of NO (11.01±0.37 nmol/mL), MDA (2.77±0.1 µmol/L), TSA (63.07±1.36 mg/dL) as well as TOC (457.80±22.48 µmolH_2O_2Eqv/L) and TAC (1.50±0.03 mmolTrolox Eqv/L) in healthy animals, respectively. The results showed statistically significant differences between the infected and healthy animals ($P<0.001$). It was concluded that the increase in levels of MDA and TOC, and the decrease in TAC might indicate the development of oxidative stress in sheep naturally infected with pox virus. It can be assumed that the increase in TSA level might be an indication of the cellular damage in the infected animals.

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INTRODUCTION

Sheep pox is a highly contagious viral infection characterized by the clinical signs of fever, conjunctivitis, rhinitis, papullous lesions in skin and mucosa, respiratory distress and death (Beytut, 2010; Plowright *et al*., 2012). The causative agent of the disease, which is classified in the Poxviridae family, can be contracted by direct contact of the skin lesions or the contaminated objects, feed or wool (Beytut, 2010). The disease is classified in the group A diseases according to the OIE due to its highly contagious nature (Mangana-Vougiouka *et al*., 1999).

Nitric oxide (NO), which mediates cytotoxic effects against pathogenic agents such as viruses, bacteria, fungi and protozoa, is synthesized from L-arginine by the catalytic action of nitric oxide synthase (NOS) (Shah *et al*., 2012; Ozcan and Ogun, 2015). In addition to its physiological properties NO plays an important role in immune system by activating macrophages. It has been well described that viral infections in general enhance NO productions. The second messenger molecule cyclic guanosine monophosphate (cGMP) can be activated by NO and hence mediates nitrosylation of the viral molecule. Due to its microbicidal function, NO is usually considered as a protective mediator in viral infections. The role of NO in viral diseases can be contributed both as a marker in the prognosis and development of vaccines and therapeutic strategies (Elanie *et al*., 2015).

Malondialdehyde (MDA), a major indicator of lipid peroxidation, is one of the most important molecules in free radical mediated cellular degenerations (Cordis *et al*., 1998; Nisbet *et al*., 2008). Degenerative changes due to lipid peroxidation can be caused by various cellular stressors, toxic substances and aging (Ozcan and Ogun, 2015). It was indicated that plasma malondialdehyde level could be used as a measure of structural and functional degenerations in cellular membranes (Nisbet *et al.*, 2008; Cigremis *et al*., 2015).

Sialic acid (SA) is an acetylated neuraminic acid derivative, and found in various tissues as a component of glycoproteins, glycolipids, oligosaccharides and polysaccharides (Karapehlivan *et al*., 2007). SA concentration is reported to increase fast in inflammatory reactions or at the beginning of cellular injuries. Therefore, total sialic acid (TSA) can be used as an indicator of tissue damage (Karapehlivan *et al*., 2014). It was previously reported that plasma SA levels could be used as an indicator of inflammatory conditions (Çitil *et al*., 2004; Erdogan *et al*., 2008).

In this study, changes at the levels of serum NO, MDA, TSA and total oxidant capacity (TOC) and antioxidant capacity (TAC) was aimed to be studied to investigate the pathogenesis of sheep pox disease in naturally infected sheep.

MATERIALS AND METHODS

Animals: The study material was 40 sheep pox infected (Group 1) and 20 clinically healthy (Group 2) Akkaraman-mixed sheep aging 1-2.5 months including both sexes. Diagnosis of the disease was based on the clinical and histopathological findings.

Determination of serum NO, MDA, TSA, TAC and TOC levels: Blood samples were collected from jugular vein and serum was separated by centrifugation at 3000 rpm for 10 minutes. NO levels were determined according to the method described by Miranda *et al*. (2001) Nitrate is reduced to nitrite by $VaCl_3$, and then in acidic environment nitrite was reacted with sulphanilamide to produce colored diazonium compound, which was read at 540 nm.

MDA concentration was measured by the method of Yoshioka *et al*. (1979) in that the reaction between thiobarbituric acid and MDA produced as an end product of lipid peroxidation. The end products were read at 535 nm.

TSA was measured colorimetrically using a spectrophotometer (UV-1201, Shimadzu, Japan) by the method of Sydow (1985) in that all bound sialic acid were separated by acid per-chloride in serum, and then the supernatants were boiled by Erlich reagent, and finally the product was read at 525 nm.

The total antioxidant capacity (TAC) was measured by a commercial kit from Rel Assay Diagnostics (Gaziantep, Turkey). The method is based on the reduction of colored 2,2'-azino-bis(3-ethylbenzotiazoline-6-sulphonic acid) (ABTS) radical to a colorless reduced form by the antioxidants that are present in the sample. The absorbance is measured at 660 nm.

The total oxidant capacity (TOC) was measured by a commercial kit from Rel Assay Diagnostics (Gaziantep, Turkey). The method is based on the principle that oxidants that are present in the sample can oxidize the ferrous ions, previously bounded to a chelator, to ferric ions. Further, in an acidic medium, the ferric ions make a colored complex with a chromogen. The intensity of the color is measured at 530 nm. The assay is calibrated with H_2O_2 and the results of the test are expressed in μmol H_2O_2 Eq/L.

Histopathology: Skin and lung samples collected from the animals were fixed in 10% neutral buffered formalin and then embedded in paraffin. Tissue sections cut from the paraffin blocks were routinely stained with Hematoxylin and Eosin (HE), and evaluated under a light microscope.

Statistical analysis: Statistical evaluation of the results was done using SPSS® (SPSS 20, IL, USA) software. The distribution of the groups was assessed by the Shapiro-Wilk test. Groups were compared with the parametric tests since the data showed normal distribution. The statistical differences between the groups were evaluated with the t-test. Results were reported as mean±SE (Standard error). In the statistical evaluation, $P<0.05$ was considered to be statistically significant.

RESULTS

Clinical signs in animals included high fever, conjunctivitis, rhinitis, serous to muco-purulent nasal discharge, lacrimation, loss of attention to environment and exhaustion. Three sheep with very poor clinical condition died during the clinical examination. Sheep pox lesions, which were characterized by irregularly shaped well-circumscribed papules in less wooled areas such as abdomen and medial thigh, were noted. Lung auscultation revealed hardened vesicular sounds. Other clinical findings were 40.27±0.97˚C body temperature, 102.6±1.84/min pulse rate, and 32.8±0.75/min respiratory rate in the infected animals as compared to 39.10±0.11˚C, 92.55±1.59/min and 24.95±0.65/min in healthy animals, respectively. Differences between the infected and healthy animals in body temperature, pulse and respiratory rates were determined to be statistically significant ($P<0.001$).

Serum levels of NO, MDA, TSA, as well as TOC and TAC values in infected and healthy sheep are given in Table 1. In all parameters differences between the infected and healthy animals were determined to be statistically significant ($P<0.001$).

In histological examination, microscopic lesions of the skin samples in the infected sheep were characterized by hydropic degeneration of the epidermis. Loosening of cellular junctions and liquefaction of the epithelia were noted. Ballooning of the Stratum spinosum epithelia mostly showing nuclear marginal hyperchromasia was seen. Few sheep pox cells having vacuolated nuclei, marginated chromatin and intracytoplasmic eosinophilic inclusions were also detected. Hyperkeratosis, parakeratosis and acanthosis accompanied the above mentioned changes (Fig. 1). In lungs, proliferative bronchiolitis and alveolitis were the main findings. Characteristic sheep pox cells were observed in the infected areas. Mild to severe congestion, monocyte and occasional neutrophil leukocyte infiltration were also noted (Fig. 2).

DISCUSSION

The causative agent of sheep pox disease is Capripox virus, belonging to the Poxviridae family. The disease is highly contagious and can be contracted directly from the infected animals or indirectly from contaminated materials. However, respiratory route is more common (Yeruham *et al*., 2007). Typical clinical signs include anorexia, high fever, increase in pulse and respiratory rates, edema in eyelids, conjunctivitis, nasal discharge, rhinitis, pneumonia, irregularly shaped swellings in skin and mucosa (Beytut, 2010; Plowright *et al*., 2012). Similarly, in the present study infected sheep showed clinical signs of high fever, rhinitis, serous to muco-purulent nasal discharge, lacrimation, loss of attention to environment and exhaustion. Hardened vesicular sounds were also noted in lung auscultation. Compared to the control group body temperature, pulse and respiratory rates were significantly higher in the infected sheep.

Skin lesions in sheep pox infection are well described elsewhere (Mauldin and Peters-Kennedy, 2016). All of the infected animals in this study showed classical skin lesions, which were mostly irregularly shaped, well demarcated, umblicated and sometimes surrounded by a hyperemic ring. These typical papullous lesions were noted especially in less wooled body regions such as abdomen and medial thigh. Microscopic view of these lesions revealed microvesicle formation as a result of hydropic degeneration and liquefaction, which were accompanied by hyperkeratosis, parakeratosis, acanthosis and sheep pox cells, characterized by vacuolated nucleus, marginated chromatin and intracytoplasmic eosinophilic inclusion bodies. The pulmonary lesions were recorded as typical proliferative alveolitis and bronchiolitis with occasional necrosis, congestion, mononuclear cellular infiltration and sheep pox cells. Dermal and pulmonary lesions in these animals were in accordance with the previous reports (Gulbahar *et al*., 2006; Beytut, 2010; Plowright *et al*., 2012).

Sialic acid is normally found in various tissues as a component of glycoproteins, glycolipids, polysaccharides and mucoproteins, and its serum concentration is known to increase in cellular degenerations (Haq *et al*., 1993; Karapehlivan *et al*., 2007). Therefore, determination of serum sialic acid level might give important clues about the course of various diseases. Recently, high sialic acid level has also been evaluated as an important inflammatory mediator in veterinary medicine (Uzlu *et al*., 2010). In the present study, serum TSA level in the infected animals (88.51±0.82) was found higher than that of control animals (63.07±1.36). The difference between the groups was also determined to be significant (P<0.001). The higher TSA levels in the sheep pox infected animals were evaluated as a result of inflammatory cellular degeneration in these animals.

Oxidative stress is known to occur when the neutralizing capacity of the body cannot compensate free oxygen radicals and the reactive oxygen metabolites. As a result, these molecules trigger cellular damage that causes structural and functional losses (Erdogan *et al*., 2008; Nisbet *et al*., 2008). Free oxygen radicals cause lipid peroxidation and yields increased MDA production (Erdogan *et al*., 2008). MDA is the most important end-product of lipid peroxidation and can be evaluated as a parameter of oxidative tissue damage (Nisbet *et al*., 2008). In many studies, increased serum MDA levels as a result of inflammation have been shown (Issi *et al*., 2008; Nisbet *et al*., 2008; Ergönül and Kontaş Akşar, 2009). Accordingly, in the present study serum MDA level in sheep pox infected animals (5.87±0.14 μmol/L) was determined to be higher than that of healthy animals (2.77±0.1 μmol/L). In addition, higher serum TOC level and lower TAC level was detected in the infected animals compared to the healthy ones. The findings of higher MDA and TOC level and lower TAC level in the sheep pox infected animals indicate that the disease induces severe oxidative damage. Overproduction of end products of lipid peroxidation such as MDA and chemically signaling molecule NO can be seen as a common phenomenon in various infections. These reactive oxygen species affect the host's cells and tissues. These host defense molecules are evidently produced to kill the intruding pathogens, which then suffer oxidative stress because of the host (Akaike and Maeda, 2000).

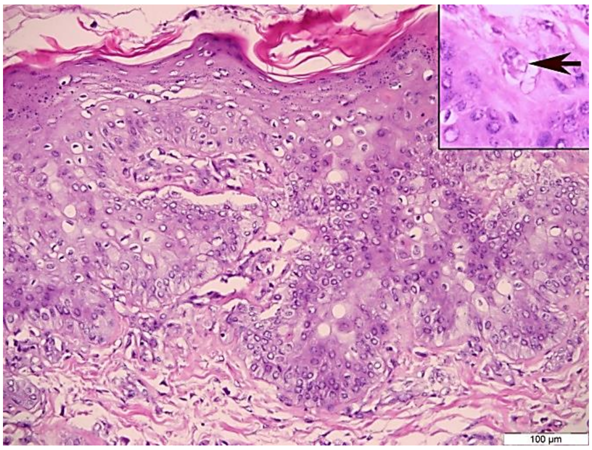

Fig. 1: Skin; Hydropic degeneration, hyper- and para-keratosis, acanthosis. Balloning of cells with nuclear marginal hyperchromasia and cellular liquefaction. Inlet figure: sheeppox cell having intracytoplasmic eosinophilic inclusion body (arrow). H&E.

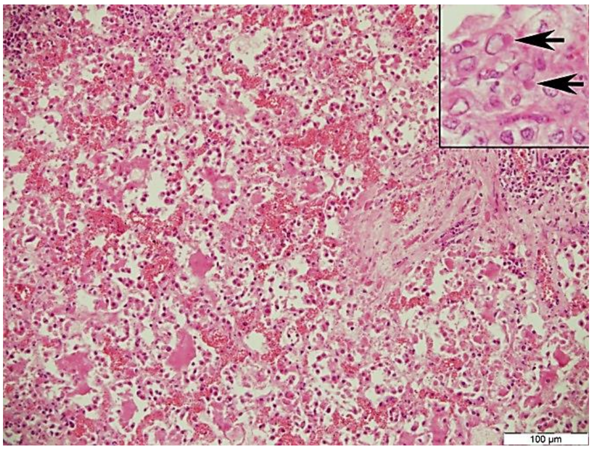

Fig. 2: Lung; Proliferative alveolitis, congestion and monocyte infiltration. Inlet figure: Histiocyte-like cells showing swollen cytoplasm, nuclear marginal hyperchromasia and intracytoplasmic eosinophilic inclusions - Sheeppox cells (arrows). H&E.

Table 1: Serum NO, MDA, TSA, TOC and TAC values in infected and healthy sheep

Groups	NO (nmol/ml)	MDA (μmol/L)	TSA (mg/dl)	TOC (μmol H_2O_2Eqv/L)	TAC (mmol Trolox Eqv/L)
Infected	36.65±1.10	5.87±0.14	88.52±0.82	685.05±10.84	1.01±0.03
Healthy	11.01±0.37	2.77±0.1	63.07±1.36	457.80±22.48	1.50±0.03

The data were given as mean±SE, P<0.001.

NO is a free radical and plays important role in many physiological and pathological processes. Bacterial endotoxins, protozoa and parasite antigens evoke NO production in macrophages, that are later mediates cytotoxic effects. A similar mechanism is also used in tumor cells. Therefore, NO can be thought to be a part of non-specific immunity (Çenesiz *et al*., 2007). In inflammatory cases, the severity of the disease increases as the capacity of the immune system is exceeded (Atakişi *et al*., 2014). Increased NO level in the infected sheep (36.65±1.10 nmol/ml) as compared to the healthy animals (11.01±0.37 nmol/ml) in the present study indicates that the virus stimulate macrophages to produce NO. Free radicals are produced primarily as effectors molecules of the host defense response. NO and oxygen radicals will provide profound insights into many aspects in clinical diagnosis of infectious diseases (Staeheli, 1990). On the other hand, NO frequently is an important mediator in intracellular inhibition of viral replication, which results in lower viral yields and more efficient host clearance of the infection, hence recovery (Carol and Takashi, 1998).

Conclusions: High serum MDA and TOC levels and low TAC level shows that oxidative stress takes place in naturally sheep pox virus infected sheep. Higher TSA level in the infected animals compared to the healthy ones is also an indicator of cellular damage in these animals.

Author's contribution: AHK, EEE and EG designed the study and performed clinical evaluation. MO and AK did biochemical analysis. HO and MK performed histopathological evaluations.

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