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Immunohistochemical Investigation of TNF-A Expression in Sheep and Goat Lung Paraffin Blocks Infected with Natural Respiratory Syncytial Virus

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Abstract

Respiratory Syncytial Virus (RSV) is an important viral agent of sheep and goat respiratory tract infections. RSV usually replicates in airway epithelium, inducing proinflammatory cytokines and chemokines. In this study, it was aimed to investigate TNF-α expression in natural RSV-infected sheep and goat lung paraffin blocks by immunohistochemical method. The material of the study consisted of 29 lung paraffin blocks (19 sheep and 10 goats), which were admitted to Etlik Veterinary Control and Research Institute with the suspicion of pneumonia from Ankara and surrounding provinces between 2015 and 2020. Histopathological findings such as degeneration and desquamation in the bronchial and bronchial epithelium, fibromuscular hypertrophy, hyperplasia in the peribronchial lymphoid tissue, cell infiltration in the interalveolar septum were common in sheep and goat lung paraffin block tissues. Immunohistochemically, RSV reaction was not statistically significant in sheep and goats in bronchial and bronchial epithelium and cell debris, bronchial glands, interalveolar septum inflammatory cells and cytoplasm of alveolar macrophages ($p > 0.05$). A statistically significant increase in TNF-α expression was determined in goat lung paraffin blocks compared to sheep ($p<0.05$).

In conclusion, an increase in TNFα expression was detected in native RSV-infected sheep and goat lung tissue. It is thought that the results of the study will contribute to the treatment of sheep and goat respiratory tract RSV infections.

Keywords; RSV, TNF-α, Immunohistochemistry, Sheep, Goat

Introduction

Respiratory Syncytial Virus (RSV) targets the respiratory system such as rhinitis, bronchiolitis, pneumonia and sometimes causes otitis media [1]. RSV is an enveloped, non-segmented, negative-stranded RNA virus in the family Paramyxoviridae [2-4]. RSVs have been defined as human RSV (HRSV), bovine RSV (BRSV), ovine RSV (ORSV), and caprine RSV (CRSV) [5-7]. RSV is the most common and important cause of lower respiratory tract infections in cattle, calves, and young infants, and clinical and patho-logical findings in BRSV-infected calves and HRSV-infect-ed infants are similar [8,9]. Sheep develop respiratory tract infections due to ORSV in the natural environment and are also susceptible to BRSV [10]. It has also been shown that BRSV, HRSV, and CRSV strains are closely related [6]. RSVs have three transmembrane glycoproteins of the 11 proteins encoded by the viral genome, and the large binding gly-coprotein (G), fusion protein (F), and small hydrophobic protein (SH) are found inside the viral envelope G (bind-

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ing protein) and F (fusion protein) encoded by RSVs are the two major surface glycoproteins [11]. Sheep RSV has the F gene and its encoded protein, bovine RSV has 85% nucleotide and 94% amino acid identity [12]. The G and F proteins play important roles in the binding and entry of HRSV and BRSV into target cells [13].

Transmission of RSV occurs through aerosol droplets of infected animals, direct contact, or indirectly through contaminated surfaces [14]. RSV proliferates in ciliary airway epithelial cells and type I and type II alveolar pneumocyte cells [15,16] and shows necrotic bronchiolitis, degenerative or necrotic changes in bronchiolar epithelium, and peribronchial lymphocyte infiltration [17,18]. Diffuse interstitial thickening of the alveolar septa is seen as a result of infiltration of inflammatory cells (ie, lymphocytes, macrophages) in the alveolar septum and type II pneumocyte hyperplasia. In the alveolar lumen, fluid with seroproteins, cell debris, a few alveolar macrophages, and occasionally neutrophils are detected [19]. Multinucleated syncytial cells are most often observed in the bronchiolar wall or lumen and less fre-quently in the alveoli [5]. Eosinophilic intracytoplasmic viral inclusions can be seen in mononuclear cells or epithelium as well as in syncytial cells [20].
Alveolar macrophages are a possible target for RSV infection [21]. In RSV infection, a rapid response develops by macrophages with the production of a wide variety of cytokines such as TNF-α, IL-1, IL-6, IL-8 [19,21,22]. Tumor necrosis factor-alpha (TNF-α) is a central mediator of immune and inflammatory responses and is important in host responses to infection [23]. In addition, TNF-α against HRSV was determined to have an antiviral effect in vitro [24,25].

In this study, it was aimed to investigate TNF-α expression in lung paraffin blocks in sheep and goat natural RSV infections by immunohistochemical method.

Material and Method

The study material consisted of a total of 29 lung archive paraffin blocks, from 19 sheep and 10 goats, which were brought to xxx Institute with the suspicion of pneumonia between 2015 and 2020. In addition, histopathological findings of interstitial pneumonia and immunohistochemical RSV positive lung paraffin blocks were used. This study was approved by the Kastamonu University Animal Experiments Local Ethics Committee (Number: E-16498365-604.01.02-2100063448 Decision No: 25/3).

Histopathological Method

Lung paraffin blocks, 5 µm thick sections with a microtome (Leica RM 2255) were taken on slides with poly-L-lysine. Sections were stained on a Hematoxylin-eosin Stainer (Shandon) and covered with a coverslip by dripping entellan. Hematoxylin-Eosin stained sections were examined under a light microscope and photographed (Leica DM 400B). Histopathology scores were classified as mild (+), moderate (++) and severe (+++) expression according to the criteria determined by Grubor, et al. [26].

Immunohistochemical Method

Sections taken from lung paraffin blocks to adhesive slides, deparaffinization and rehydration procedures were applied. Immunohistochemical staining was performed according to the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit-Micro polymer (ab236466) kit procedure. Hydrogen peroxide was dripped onto the sections. Proteinase K (ab64220) was added for antigen retrieval treatment and incubated at room temperature. The protein block solution was poured and left for 10 minutes and then incubated in primary antibody (1:100 anti Mouse RSV monoclonol (Cat NO: ab 43812), 1:350 anti- Rabbit TNF-α (Cat NO: ab 6671)) for 1 hour at room temperature. The Mouse Detection Reagent (Supplementary) solution was incubated for 10 min followed by a drop of Goat anti-rabbit HRP-conjugate for 15 min. DAB chromogen was added to demonstrate antigen-antibody reaction. Background staining was done with Mayer's hematoxylin. It was passed through xylol and alcohol series and closed with a coverslip by dripping enthalen. Sections were examined under a light microscope and photographed (Leica DM 400B). Immunohistochemical staining scores were classified as mild (+), moderate (++) and severe (+++) expression according to the criteria determined by Yavuz and Dinçel [27].

Statistics

Histopathological and immunohistochemical score in sheep and goat paraffin blocks were evaluated using IBM SPSS Statistics 25.0 software and Mann-Whitney's U test. The criterion for statistical significance was $p<0.05$. All values are presented as mean±standard deviation.

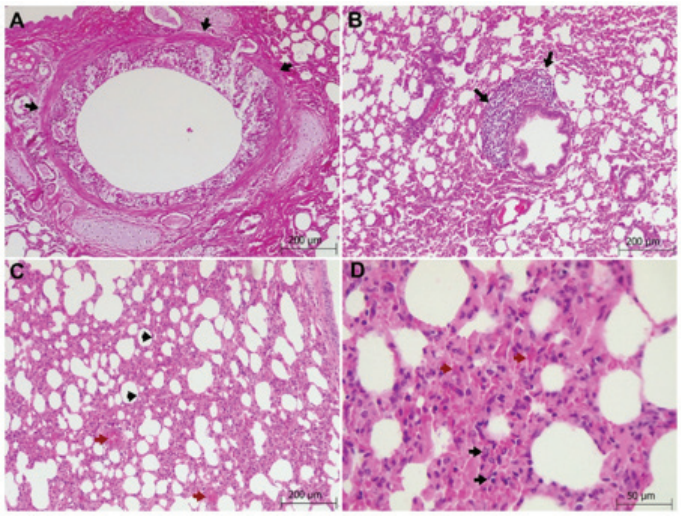
Results

Histopathological Results

Histopathological findings of RSV positive cases in sheep and goat lung paraffin blocks were scored by semiquantitative method. Histopathological findings such as degeneration and desquamation in the bronchi and bronchiolar epithelium, fibromuscular hypertrophy (Fig. 1A), hyperplasia in the peribronchial lymphoid tissue (Fig. 1B), cell infiltration in the interalveolar septum (Fig. 1 C-D) were not statistically significant ($p> 0.05$) in sheep and goat lung paraffin block tissues (Table 1). It was determined that al-

veolar macrophages increased slightly in sheep and moderately increased in goats. In the interalveolar septum, syncytial giant cells were detected in 15 cases in sheep and 8 cases in goats.

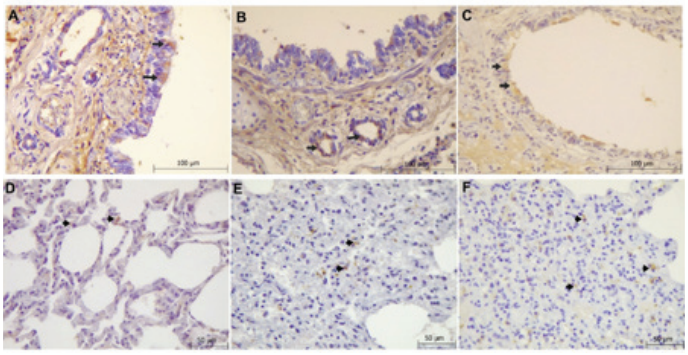
Immunohistochemical Results



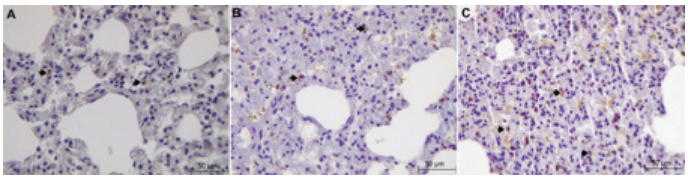
Figures 1. Hematoxylin-Eosin Staining. Lung tissue of sheep and goat. A. Moderate fibromuscular hypertrophy (black arrows) and bronchial gland hyperplasia. Bar:200 µm. B. Moderate peribronchiolar lymphoid tissue hyperplasia (black arrows), cell infiltration, and hyperemia in the interalveolar septum. Bar:200 µm. C. Moderate cell infiltration (black arrows) and hyperemia (red arrows) in the interalveolar septum. Bar:200 µm. D. Severe cell infiltration (black arrows) and hyperemia in the interlveolar septum. Bar: 50 µm.

In the Immunohistochemistry, RSV was found positive in lung paraffin block tissue of 19 sheep and 10 goats. Accordingly, RSV reaction in sheep and goat lungs, cell debris in bronchial and bronchiolar epithelium, and cell debris in lumen, bronchial glands and alveolar macrophages were statistically similar ($p>0.05$). While RSV antigen was found in the bronchial epithelium and cell debris in the lumen mild in 21% of and severe in 10% of sheep (Fig 2A), RSV antigen was not found in the bronchial epithelium and cell debris in the lumen of goats. RSV antigen was detected in bronchiol epithelium and cell debris in the lumen, mild (15% of sheep and 20% of goats) and severe only in 10% of sheep (Fig 2C). RSV antigen positive in bronchial glands was found only in one sheep (Fig 2B). Interalveolar septum cell infiltrations to RSV antigen in mild (31% of sheep and 50% of goats Fig 2D), moderate (21% of sheep, Fig 2E) and severe (15% of sheep and 20% of goats Fig 2F) was seen. In alveolar macrophages, RSV antigen was detected mild (57% of sheep, 60% of goats), moderate (15% and 10% of sheep), severe (15% of sheep and 30% of goats).

TNF-α expression in alveolar macrophages was mild (12 sheep and 3 goats Fig 3A), moderate (1 sheep and 3 goats Fig 3B), severe (2 sheep and 3 goats Fig 3C). It was determined that the expression of TNF-α in sheep and goat lung paraffin blocks was statistically more intensely stained ($p<0.05$) in goats than in sheep (Fig 4, Table 1).



Figures 2. Immunohistochemical staining of sheep and goat lung's. A. Severe anti-RSV reaction in the bronchial epithelium (black arrows). Bar: 100µm. B. Anti-RSV reaction (black arrows) in the bronchial glands. Bar: 100µm.C. Anti-RSV reaction in the bronchiolar epithelium (black arrows) and alveolar lumen. Bar: 100µm. D. Mild anti-RSV positive reaction (black arrows) in the cytoplasm of interalveolar septal cell infiltrates. Bar: 50µm. E. Moderate anti-RSV positive reaction (black arrows) in the interalveolar septum cell cytoplasm. Bar: 50µm. F. Severe anti-RSV positive reaction (black arrows) in the interalveolar septum cell cytoplasm. Bar: 50µm.



Figures 3. Immunohistochemical staining. Lungs. Sheep and goat. A. Mild anti-TNF-α positive reactions (black arrows) in the interalveolar septum cell cytoplasm. Bar:50µm. B. Moderate anti-TNF-α positive reaction (black arrows) in the interalveolar septum cell cytoplasm and alveolar macrophages. Bar: 50µm. C. Severe anti-TNF-α positive reaction (black arrows) in the interalveolar septum cell cytoplasm. Bar: 50µm.

Table 1. Statistical findings of histopathological and immunohistochemical staining scores in RSV antigen positive sheep and goat lung blocks.

	Lesions	Sheep	Goat	*P value*
Histopathology	Degeneration and desquamation of the bronchial and bronchiolar epithelium	1,21±0.91	1.50±0.84	0.438
	Fibromuscular hypertrophy	1.15±0.68	1.30±0.67	0.594
	Lymphoid hyperplasia	0.63±0.88	0.60±0.84	1.00
	Thickening of the interalveolar septum	1.89±0.65	1.50±0.53	0.122
	Epithelialization	1.00±0.33	1.20±0.63	0.242
	Hyalin membrane	0.84±0.37	1.10±0.56	0.162
	Intraalveolar edema	0.21±0.53	0.70±0.94	0.118
	Alveolar macrophage	1.89±0.93	2.10±0.56	0.496
	Hiperemi	1.42±0.60	1.20±0.42	0.334
IHC	In the bronch epithelium and cell debris	0.52±0.96	0.00±0.00	0.510
	In bronchial epithelium and cell debris	0.42±0.96	0.20±0.42	0.845
	In the Bronchial Glands	0.36±0.95	0.00±0.00	0.193
	In inflammatory cells in the interalveolar septal tissue.	1.10±0.99	1.10±1.10	0.903
	Alveolar macrophages	1.36±0.89	1.70±0.94	0.379

Wan Witney U testi. Mean+Std. $P<0.05$.

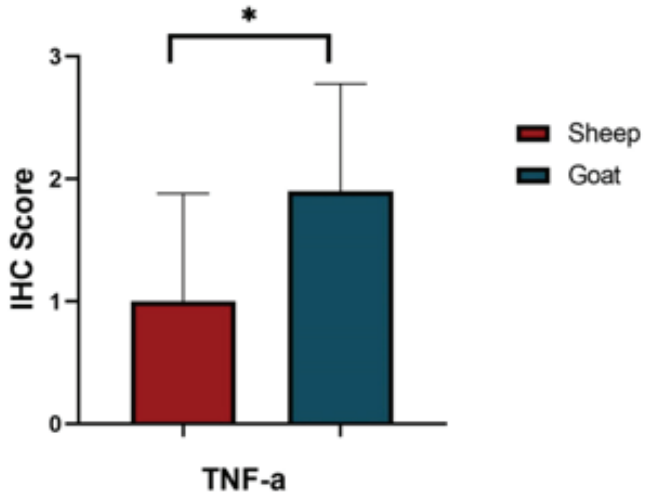


Figure 4. TNF-α expression increased significantly in goat lung paraffin tissue compared to sheep ($p<0.05$). Wan Witney U test. Mean+Std. *$p<0.05$.

Discussion

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in humans and animals. RSV causes epidemics in ruminant herds and causes serious economic losses. RSV outbreaks usually occur in autumn and winter [9]. BRSV and HRSV proliferate in ciliary airway epithelial cells and type II pneumocytes [15,17] and innate immunity is activated, resulting in the induction of proinflammatory cytokines and chemokines [19,28,29]. In humans, calves and lambs, cytokines such as TNF-α, IL-6 and IL-8 increase gene expression from alveolar epithelium and alveolar macrophages [30-32]. We investigated TNF-α expression in natural RSV-infected sheep and goat lung paraffin tissue by immunohistochemical method.

In RSV infections, cytokines and chemokines induce adhesion molecules [19,28], neutrophils and lymphocytes proliferation, resulting in bronchiolitis and interstitial pneumonia in the lung [29]. Clinical symptoms such as fever, weakness, mucoid nasal discharge, continuous and deep breathing, tachypnea, cough, and anorexia are seen in RSV infections [17,29,33,34]. Necropsy findings are similar in RSV infections of ruminants. Macroscopically, irregular lobular or fused atelectasis areas and consolidation areas localized in the cranioventral region of the lung are seen [35,36]. In all lobes can be seen interstitial edema, while emphysema is detected in the diaphragm lobes [37]. Also, the trachea, bronchi, and bronchioles may contain mucopurulent discharge [5,17]. In RSV infections, histopathologically, bronchitis, bronchiolitis, thickening of inflammatory cell infiltrates in the alveolar septum, lymphocyte accumulations in the perivascular and peribronchiolar spaces, hyperplasia in the epithelial cells of the bronchi and bronchioles are caused [35]. There are also neutrophils and macrophages in the lumen of the bronchi, bronchioles and alveoli and multinucleated syncytial cells and hyaline membranes [32]. Similar to previous studies, in the paraffin tissue of the naturally RSV virus-infected sheep and goat lungs were detected degeneration and desquamation of the bronchial and bronchiolar epithelium, fibromuscular hypertrophy, lymphoid hyperplasia, cell infiltration in the interalveolar septum, epithelialization, and hyaline membrane such as interstitial pneumonia findings. Interstitial pneumonia findings in sheep and goat lung paraffin blocks were not statistically significant ($p>0.05$) and RSV infections were similar in sheep and goats.

RSV replication occurs in both non-ciliary cells of the bronchiole and types II cells at the bronchiole/alveolar junction, where airflow and gas exchange is greatly impaired due to cell damage and inflammation in these areas [38]. In the study, it was determined that RSV antigen in the bronchial epithelium was mild in 21% of sheep and severe in 10%, and RSV antigen was not found in the bronchial epithelium of goats. It was detected in the lung paraffin blocks of sheep and goats with mild severity in the bronchiolar epithelium (15% and 20%, respectively) and severe only in 10% of sheep. In both humans and lambs, RSV viral antigen was detected in bronchial and bronchiolar epithelial cells, as well as in type II pneumocytes [15,18]. In this study, RSV antigen was detected in sheep and goat lung bronchial and bronchiolar epithelial cells, similar to RSV antigen determination studies in humans and lambs. According to Viuff, et al. [16] found that although the number of mature alveolar macrophages increased during experimental BRSV infection in calves, they only rarely contained RSV antigen. In the study, generally mild RSV reaction was determined in alveolar macrophages in sheep and goats.

TNF-α has an important role in the pathogenesis of the disease [32]. TNF-α is a 17-kd, 157-amino acid cytokine secreted by many cells, including macrophages, monocytes, T cells, natural killer cells, and neutrophils [39]. Franke, et al. [40] found that RSV-infected macrophages released TNF-α in a virus dose-dependent manner in a study inoculating murine alveolar macrophages with RSV. Ryntved, et al. [32] identified elevated TNF-α levels on days when severe lung lesions and clinical manifestations were exacerbated and BRSV-antigen levels were highest. In the study, TNF-α expression in alveolar macrophages was mild (12 sheep and 3 goats-), moderate (1 sheep and 3 goats-), severe (2 sheep and 3 goats). In addition, TNF-α expression in the cytoplasm of interalveolar septal cell infiltrates was found to be significantly higher ($p<0.05$) in goats compared to sheep. These findings show that natural RSV infections of sheep and goats cause in the lung increased expression of TNF-α.

Conclusion

Viral infections cause the release of many proinflammatory cytokines in the lung tissue. In this study, an increase in the expression of the proinflammatory cytokine TNF-α was detected in the lung tissue of naturally RSV infected sheep and goats. In addition, the results of the study are thought to contribute to the treatment of RSV infections in ruminants.

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