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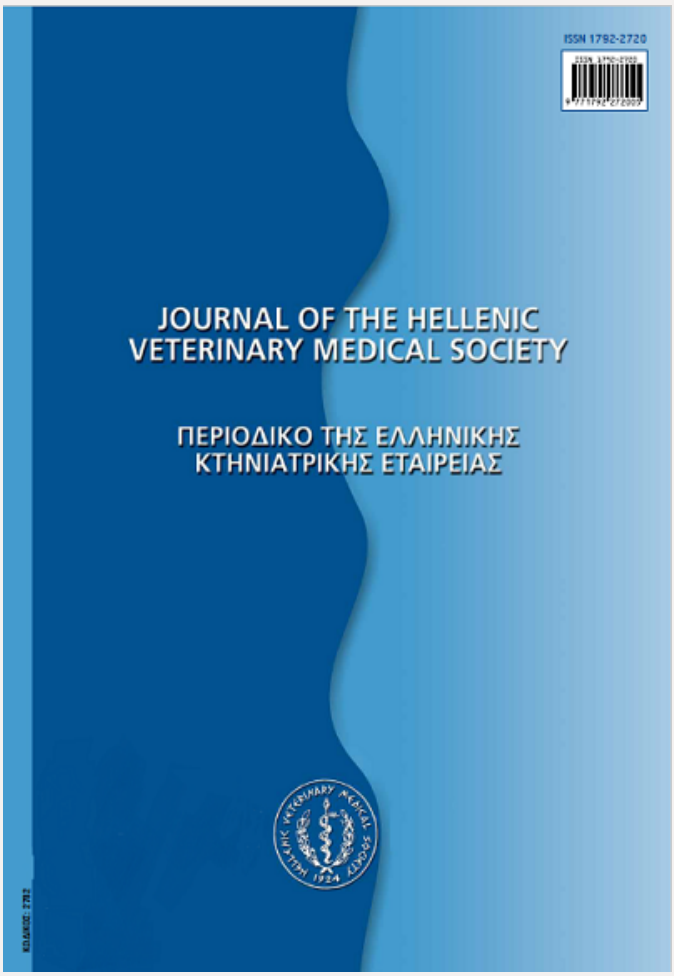

The evaluation of oxidative stress in lambs with Pestivirus infection

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Research article
Ερευνητικό άρθρο

The evaluation of oxidative stress in lambs with *Pestivirus* infection

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ABSTRACT. This study was carried out to measure the plasma malondialdehyde (MDA) level, and erythrocyte glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) activities in lambs with *Pestivirus*. Sixty lambs aged between 2.5-3 months old were included in the study. Blood and faeces samples were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) test, using pan-Pestivirus primers. Clinical and virological examinations revealed that the numbers of non-infected and *Pestivirus* -infected animals were 20 (Group 1) and 40 (Group 2), respectively. Plasma MDA levels were found significantly higher in lambs with *Pestivirus* than that of controls ($p<0.05$) whilst the erythrocyte GSH-Px, CAT and SOD activities were significantly lower in lambs with *Pestivirus* ($p<0.05$) than in the controls. The determination of increased plasma MDA levels and decreased erythrocyte GSH-Px, CAT and SOD activities in the infected lambs confirms that oxidative stress is observed in *Pestivirus*. The presence of oxidative stress in *Pestivirus* indicates that the equilibrium between oxidants and antioxidants is shifted towards oxidants in lambs with *Pestivirus*.

***Key words*:** lamb, oxidative stress, *Pestivirus*

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INTRODUCTION

The *Pestivirus* genus of the *Flaviviridae* includes *Border disease virus* (BDV), *Bovine viral diarrhea virus-1* (BVDV-1), BVDV-2 and *Classical swine fever virus* (CSFV). These viruses are structurally and antigenically closely related, and cross infections between species may occur (O'Neill et al. 2004). Pestiviruses can infect animal species other than their natural hosts but, historically, they were named according to the animal from which they were isolated. Analysis of virus isolates by cross neutralisation, monoclonal antibody (mAb) typing and sequence analysis has shown that classification based on virus characteristics is suggested (Valdazo-Gonzalez et al. 2007).

Clinical signs in *Pestivirus* infected sheep vary and include barren ewes, abortions and stillbirths. Affected lambs have been reported to exhibit clonic muscular tremors and other neurological signs, growth retardation, skeletal malformation, weakness, low neonatal survival rate, abnormal fleece or pigmentation in fine- and medium-fleeced breeds of sheep (Nettleton et al. 1998; García-Pérez et al. 2009). Pestiviral infections results in major economic losses to sheep industry as well as in swine and cattle populations (Sweasey et al. 1979; Wyovet 2003). In addition, it may exacerbate the severity of other infections (e.g. *Escherichia coli*, *Cryptosporidium* sp. and *Mamheimia haemolytica*), compromising the immune response to other pathogens (Hussin-Woldehiwet 1994; Campbell 2004; García-Pérez et al. 2009). Infection of pregnant sheep with *Pestivirus* may result in birth of persistently infected (PI) lambs (Nettleton et al. 1998). The control of the *Pestivirus* infection is possible through immediate identification and culling of the PI animals that are the main infection source in a flock (Nettleton et al. 1998). The diagnosis of the healthy-looking PI sheep infected through congenital transmission can be done by direct detection of viral antigen or viral RNA in leukocytes, or by isolation of non-cytopathic virus from blood or serum in laboratory cell cultures (Nettleton et al. 1998).

Recent studies indicate that infections increase the production of free radicals (Suntres et al. 2002; Gurgoze et al. 2003; Kumaraguruparan et al. 2003), which are constantly formed in biological systems and removed by enzymatic and non-enzymatic defence mechanisms. Under normal conditions, the production and the elimination of free radicals are in a dynamic equilibrium. This balance may be disturbed when the generation of free radicals becomes higher than the production capacity of systemic antioxidant defence (Halliwell-Gutteridge 1999) or deficiency of counteracting antioxidant system. The impaired equilibrium in favour of oxidants is called oxidative stress and is implicated in the pathogenesis of many diseases and inflammatory conditions (Romero et al. 1998; Halliwell-Gutteridge 1999).

Superoxide dismutase, GSH-Px and CAT enzymes can hinder both the accumulation of free radicals and the initiation of lipid peroxidation (Gutteridge 1995, Kohen-Nyska 2002). The severity of oxidative degradation can be identified by measuring the levels of the end products like MDA formed as a result of lipid peroxidation, and the antioxidant enzyme activity in the blood and tissues (Yagi 1998, Kohen-Nyska 2002).

Evidence is growing on the role of reactive oxygen species (ROS) in the pathogenesis of several viral diseases caused by *Human immunodeficiency virus* (HIV), *Hepatitis C virus* (HCV), *Feline immunodeficiency virus* (FIV) and *Canine parvovirus*, reflecting an increased interest to further study the role of free radicals in other virus infections (Treintinger et al. 2000; Gil et al. 2003; Webb et al. 2008; Panda et al. 2009; Sun et al. 2013). Although Schweizer and Peterhans (1999) reported the crucial impact of oxidative stress on the death of bovine turbinate cells caused by cytopathic BVDV, oxidative stress has not been investigated in lambs with *Pestivirus*. Therefore, this study was performed to determine the plasma MDA level, erythrocyte GSH-Px, CAT and SOD activities in lambs with *Pestivirus*.

MATERIALS AND METHODS

Animals and Grouping of Lambs

A 2.5 to 3-month-old Akkaraman lamb with a history of haemorrhagical diarrhea, tremor, inability to stand unaided and gait anomaly was admitted to the Department of Internal Medicine, Faculty of Veteri-

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nary Medicine at Erciyes University in Turkey. The owner reported abortions in the flock during the breeding season. Other symptoms of the affected lambs included diarrhea, tremors, falling down and unable to stand, and severe contractions of the leg muscles and death of many lambs for the last two weeks.

The affected flock comprised of 196 animals, of which 40 affected lambs were used in this study. In addition, samples were collected from 20 healthy and same breed lambs aged between 2.5 and 3 months old from another pen managed the same way as the affected animals. The prophylactic routine treatment of all animals at admission included desparasitation. Based on the serum neutralization test results performed against the NADL strain of BVDV, Group 2 were divided into two subgroups as antibody positive (22 animals) and antibody negative (18 animals).

Blood sampling

Blood samples from the lambs were collected into tubes with heparin for determination of plasma MDA levels, erythrocyte GSH-Px, CAT and SOD activities. The blood samples collected to tubes with caoline and EDTA were used for the cell culture and definite diagnosis using RT-PCR. The samples of the blood as well as the faecal samples obtained from the lambs were examined by RT-PCR test using pan-pestivirus primers. The definitive diagnosis of the disease was based on the positive clinical and virological examinations.

Virological Procedure

Cell culture and virus isolation

Madin-Darby bovine kidney (MDBK) cell culture (obtained from the Cell Culture Collection of the Ankara University Virology Laboratory, Turkey) was prepared for virus isolation and the serum neutralization tests, respectively. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco®, Invitrogen, USA) supplemented with 10% fetal bovine serum (Biological Ind. ®, Israel).

Blood samples collected from diseased lambs were prepared for inoculation. Inoculated cell cultures were checked microscopically for cytopathic effects (CPE) daily. If a CPE was not observed, a further passage of the infected cells was performed (Fenner et al. 1987).

RT-PCR

RT-PCR was applied on biological specimens (leukocytes, faeces and swap) from the lambs and on the cell-culture supernatant. Viral RNA extracted with guanidium/phenol/chloroform-isoamyl alcohol methods from *Pestivirus* positive lambs (Chomczynski-Sacchi 2006). Synthesis of cDNA from extracted RNA was carried out, using RevertAid™First Strand CDNA Synthesis Kit (Fermentas®, Lithuania). The primers chosen from the 5'untranslated region (5'-UTR) of the BVDV genome were used for amplification of expected 288 bp fragment as previously described by Vilček et al. (1994).

The amplification step was carried out, using the following thermal cycles: the denaturation step at 95 °C for 5 min; 40 cycles of the amplification step (denaturation at 95 °C for 1.30 min, annealing at 45 °C for 1 min, and extension at 72 °C for 40 sec), and a final extension step at 72 °C for 10 min. The PCR products were resolved by gel electrophoresis in 1.7% agarose gel in TBE (Tris-boric acid-EDTA) buffer, stained by ethidium bromide. The product of the RT-PCR was visualized using a UV transilluminator. Gel images were recorded digitally with the Kodak Gel Doc 2000 system (Bio-Rad Laboratories®, NY, USA).

Sequence and phylogenetic analysis

In order to confirm RT-PCR results amplicons from all positive isolates were gel purified by a commercial kit (High Pure PCR product purification kit, Roche). The amplified fragments were sequenced in ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) in both directions using the amplification primers. Sequence results were analysed online for BLAST and recorded with the accession number of KX388379 at Genebank.

Serological procedure

Serum neutralization test

Serum neutralization (SN) test was performed in the MDBK cell cultures grown in 96-well microplates for specific *Pestivirus* antibodies. In brief, the neutralizing titers of each of the five-fold diluted serum were determined against the NADL strain of BVDV (100 TCID50 in 0.05 ml) in wells. The plates were incubated at 37 °C in an incubator supplied with 5% CO2.

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The CPE was examined with an inverting microscope after 72 h. The SN titers were expressed as the reciprocal of the highest serum dilution that neutralized the virus at the 50% end point.

Measurement of oxidative stress indices

Blood samples were separated into plasma and erythrocytes. Erythrocytes were prepared according to Witterbourn et al. (1975). Plasma MDA levels were determined as described by Yoshioka et al. (1979) whilst erythrocyte haemoglobin levels were identified, using the methods published by Fairbanks and Klee (1987). Measurement of the GSH-Px, CAT and SOD activities were performed according to Paglie and Valentie (1967), Luck (1955) and Sun et al. (1988), respectively.

Statistical analyses

Data were analyzed, using Mann-Whitney U, one-way ANOVA and Duncan tests. Statistical significance was attained at $p<0.05$ level. Data were expressed as mean values ± SEM. Statistical comparisons were carried out, using SPPS 13.0 statistical software.

RESULTS

According to the anamnesis and clinical examination results, no infection other than *Pestivirus* was found in the examined lambs.

None of the sampled animals exhibited hairy fleece or abnormal body pigmentation. The control group was assigned as uninfected by means of the clinical and the virological examinations. The cell culture virus isolations showed that 17 of the 40 infected samples exhibited CPE. Using RT-PCR, non-cytopathic strain was determined in cell culture supernatants of the 23 lambs with no CPE. While the SN tests revealed that 22 of the 40 serum samples were antibody positive, neutralizing homologous antibody titers against to NADL virus ranged between 0 and 80. Nine of the non-cytopathogenic samples (23 animals) were antibody negative. For each supernatant, a fragment of the 5´UTR was amplified by RT-PCR, observed as a single band of the 288bp specific positive products (Figure 1).

Plasma MDA level was significantly higher in the lambs with *Pestivirus* compared to controls ($p<0.05$). However, the erythrocyte GSH-Px, CAT and SOD activities in the lambs with *Pestivirus* were significantly lower ($p<0.05$) than those of the controls (Table 1).

The same trends in these results were observed for the antibody positive and negative sick lambs (Table 2).

DISCUSSION

Pestivirus infection in sheep is characterized by infertility, abortion, abnormal fleece and body condition, tremors and other neurological symptoms. The newborn lambs are weak, and usually have low life expectancy and growth performance (Valdazo-Gonzalez et al. 2008; García-Pérez et al. 2009). Similarly, the clinical observations of the *Pestivirus* positive lambs in the current study showed weakness, clonic muscle tremors and diarrhea.

Previous research attempts emphasised that free radicals play an important role in pathogenesis of various diseases including infections, cardiovascular system diseases, skin diseases, genetic and metabolic disorders, autoimmune diseases, cancer, allergic diseases,

Table 1.Plasma MDA levels and erythrocyte GSH-Px, CAT and SOD activities in control and in lambs with *Pestivirus* as mean values ± SEM

Parameter	Group 1 (n: 20)	Group 2 (n: 40)
MDA (nmol/mgHb)	0.75±0.23	1.27±0.23*
SOD (U/mgHb)	0.63±0.13	0.51±0.16*
CAT (k/gHb)	11.64±5.09	8.08±3.14*
GSH-Px (μmol $NADPH^+$/gHb)	11.41±1.90	7.01±3.98*

*The difference between groups coded with asterisk in the same row is significant ($p<0.05$).

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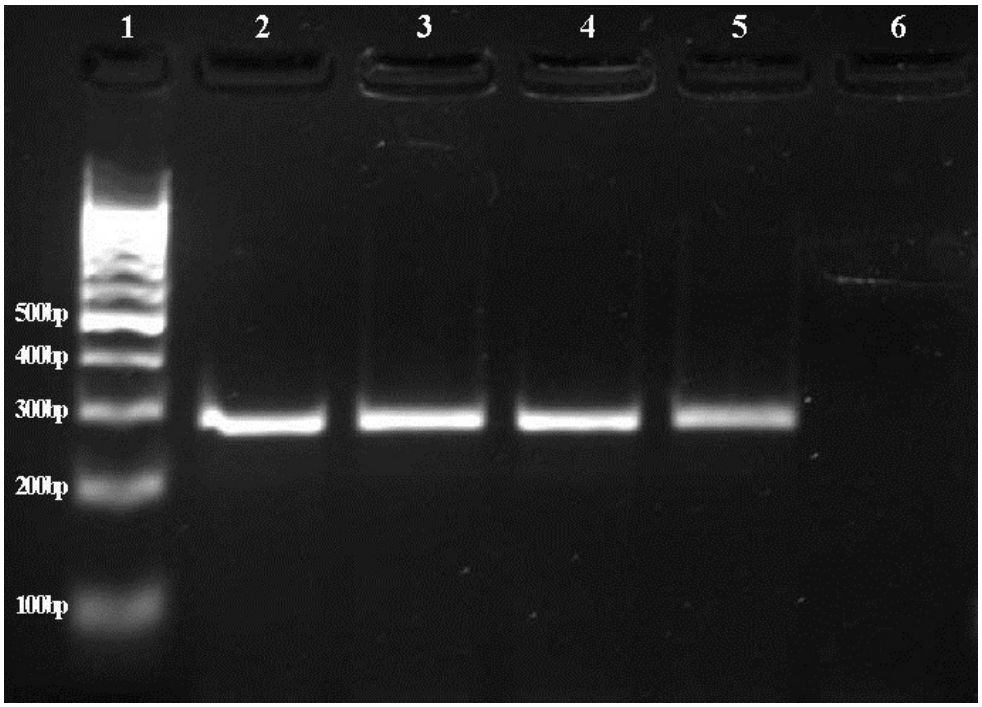
Figure 1. Electrophoresis of RT-PCR products covering the virus genome. Lanes 1:100bp DNA Ladder, 2: + Control NADL, 3: Leukocyte, 4: Faeces, 5: Swap, 6: Control



neurodegenerative diseases, rheumatoid arthritis, Parkinson, eye diseases, HIV, hepatitis C, sheep-pox (Çam et al. 2009; Halliwell-Gutteridge 1999; Romero et al. 1998). This study contributes to the existing literature through quantification of lipid peroxidation levels and changes in antioxidant enzyme activity in lambs with *Pestivirus* infection for the first time.

Lipid peroxidation gives rise to the production of many reactive electrophiles (e.g. epoxides and aldehydes). Some of these products interact with protein and DNA, resulting in toxic and mutagenic consequences. MDA is the major product of lipid peroxidation and influences the ion exchange in cell membranes. It may trigger cross-linking of the compounds on cell membrane, resulting in changes in ion permeability and enzyme activity. It has been shown to be mutagenic due to its reaction with DNA nitrogen bases, in addition to being genotoxic and carcinogenic for cell cultures (Marentt, 2002). Many studies exist investigating the relationship between lipid peroxidation biomarkers and viral infections. While some studies indicate that MDA levels increase in humans with HIV (Jareno et al., 1998; Gil et al. 2003; Ogunro et al. 2005), other studies report that the MDA level is unchanged in cats with FIV (Webb et al. 2008). Sun et al. (2013) found elevated levels of MDA in humans with *hepatitis C virus*. Higher MDA concentrations than controls were reported from different animals such as dogs with parvovirus enteritis (Panda et al. 2009), ruminants with Peste des petits (PPR) (Nisbet et al. 2007) and goats with BD (Balıkçı et al. 2013), supporting the similar findings in this study. The increased levels of MDA in animals infected with *Pestivirus* indicate the presence of lipid peroxidation. Given the mutagenic characteristic of MDA, the damage in foetus with *Pestivirus* may be influenced by the generation of MDA.

The present study results demonstrated lower levels of erythrocyte CAT, SOD and GSH-Px in the in-

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Table 2. Plasma MDA levels and erythrocyte GSH-Px, CAT and SOD activities in control and in lambs antibody (+) and (-) with *Pestivirus* as mean values ± SEM

Parameter	Group 1 Control (n: 20)	Antibody (+) (n: 22)	Antibody(-) (n: 18)
MDA (nmol/mgHb)	0.75±0.23 [a]	1.24±0.20 [b]	1.31±0.27 [b]
SOD (U/mgHb)	0.63±0.13 [a]	0.52±0.18 [b]	0.48±0.12 [b]
CAT (k/gHb)	12.11±4.18 [a]	8.26±3.10 [b]	7.97±3.24 [b]
GSH-Px (μmolNADPH+/gHb)	11.41±1.90 [a]	7.03±4.33 [b]	6.88±3.44 [b]

a,b: The difference between the groups coded with different letters in the same row is significant ($p<0.05$).

fected lambs relative to the control group ($p<0.05$). SOD, GSH-Px and CAT enzymes prevent the occurrence of free radicals and initiation of lipid peroxidation (Romero et al. 1998; Halliwell-Gutteridge 1999). In a study conducted by Webb et al. (2008), SOD and GSH-Px levels increased in cats with FIV. Varying levels of erythrocyte GSH-Px and SOD concentrations were previously reported from humans with HIV (Treintinger et al. 2000; Gil et al. 2003). Lower SOD and GSH-Px concentrations found by Balıkçı et al. (2013) in goats with BD compared to controls align with the results presented in the current study. The main attribute of the lower levels of erythrocyte CAT, SOD and GSH-Px in *Pestivirus* -infected lambs may be that the antioxidant defence mechanism deteriorates vis-à-vis increased oxidative stress, in addition to the severe damage on erythrocyte membrane and other cellular structures.

CONCLUSIONS

In conclusion, elevated levels of plasma MDA and decreased erythrocytes GSH-Px, CAT and SOD activities in the infected lambs suggest that *Pestivirus* causes oxidative stress, reflecting the impaired equilibrium between oxidants and antioxidants in favour of oxidants. Further studies investigating the impacts of oxidative stress biomarkers on mutagenic consequences due to *Pestivirus* infections are warranted. To the best of our knowledge, this is the first study reporting the presence of oxidative stress in lambs with *Pestivirus*.

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