Clinical, haematological and antioxidant status in naturally poxvirus infected sheep

M. ISSI[1*], Y. GUL[1], S. YILMAZ[2]

[1] *Department of Internal Diseases, Faculty of Veterinary Medicine, Firat University, 23119 Elazig, TURKEY.*
[2] *Department of Biochemistry, Faculty of Veterinary Medicine, Firat University, 23119 Elazig, TURKEY.*

** Auteur chargé de la correspondance : E-mail : missi@firat.edu.tr*

SUMMARY

The aim of the present study was to investigate clinical and haematological findings, and antioxidant status (β-carotene, vitamins A, E, C, reduced glutathione (GSH) concentrations vs. plasma malondialdehyde (MDA) concentrations and blood catalase activity) in sheep with spontaneous pox disease stemming from an infected herd (morbidity: 18%). In this study, blood samples were collected from 20 sick sheep, clinically diagnosed for poxvirus infection (test group) and 20 healthy sheep (control group) after clinical examination. Haematological parameters were determined using a blood cell counter whereas plasma or serum vitamin A, E, C, β-carotene, MDA and GSH concentrations as well as catalase activity were measured spectrophometry. The diseased sheep exhibited significantly elevated body temperature, heart and breathing rates whereas rumen movements were markedly reduced compared to healthy controls. In parallel, haemoglobinemia and haemoglobin load in erythrocyte (MCHC) were also significantly decreased but the most severe alteration concerned thrombocytes whose counts were dramatically depressed. Marked increases of plasma MDA concentrations and catalase activities associated to significant reductions of concentrations of some antioxidants (glutathione, vitamin E and C) revealed the occurrence of an oxidative stress and membrane lipid peroxidation in sick animals. These results suggest that the poxvirus infection induced an exacerbated free radical formation and consumption of the principal antioxidant systems.

Keywords: Pox virus, antioxidant vitamins, glutathione, free radicals, clinical and haematological parameters.

RÉSUMÉ

Infection spontanée du mouton par le poxvirus : signes cliniques et hématologiques, altération du statut anti-oxydant

L'objectif de cette étude est de déterminer les signes cliniques et hématologiques ainsi que le statut anti-oxydant (concentrations en β-carotènes, vitamines A, E, C et en glutathion vs. concentration en MDA et activité plasmatique de la catalase) de moutons atteints d'une infection par le poxvirus issus d'un troupeau infecté (morbidité 18%). Au cours de cette étude, des échantillons sanguins ont été recueillis sur 20 moutons pour lesquels le diagnostic clinique a été établi (groupe test) et sur 20 moutons cliniquement sains issus d'un élevage indemne (groupe contrôle). Les paramètres hématologiques ont été déterminés grâce à l'utilisation d'un compteur cellulaire tandis que les concentrations en β-carotènes, vitamines A, E, C, glutathion et en MDA et l'activité plasmatique de la catalase ont été mesurées par spectrophotométrie. Les moutons malades ont présenté des élévations significatives de la température interne, des rythmes cardiaque et respiratoire par rapport aux contrôles alors que la motricité ruminale a été notablement réduite. Parallèlement, l'hémoglobinémie et la charge érythrocytaire en hémoglobine (CCMH) ont aussi été significativement diminuées et la numération plaquettaire s'est révélée considérablement effondrée. Les augmentations importantes des concentrations plasmatiques de MDA et de l'activité de la catalase associées à des réductions significatives des concentrations en anti-oxydants (glutathion, vitamines E et C) ont révélé l'existence d'un stress oxydatif et d'une peroxydation lipidique membranaire. Ces résultats suggèrent que l'infection par le poxvirus a induit la formation exacerbée de radicaux libres et la consommation des principaux systèmes anti-oxydants.

Mots-clés : Poxvirus, vitamines anti-oxydantes, radicaux libres, glutathion, paramètres cliniques et hématologiques.

Introduction

Sheep pox is an acute viral infection characterized by widespread pox lesions in skin and mucosa, inducing a contagious disease with fever that causes economic losses because of abortion and stillbirth [2, 15, 23, 32]. It was reported that the disease is present in Africa, North of Equator, Turkey, Middle East, India, Nepal and a part of China but not in North and South America and Australia [5, 15, 22, 23]. The causative agent of the disease is the Capripoxvirus which belongs to the family Poxviridae and spreads by inhalation. Additionally, the disease can be contacted mechanically via contact with infected animals, insect bites and contaminated articles or experimentally by intradermal or subcutaneous injections [2, 15, 22, 24]. In natural infections, the incubation period lasts 4-8 days [5, 22, 24]. Vaccination is stated to be effective to reduce the losses from poxvirus infected sheep in endemic area, and several modified live virus vaccines have been used to protect from the disease [4, 22, 24].

Free radicals are very reactive chemical species, can cause oxidation injury to the living beings by attacking the macromolecules like lipids, carbohydrates, proteins and nucleic acids. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defence systems used by organisms to deactivate and protect themselves against free radical toxicity [13, 29]. Impairment in the oxidant/antioxidant equilibrium creates a

condition known as oxidative stress. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases [7, 11, 12]. Both oxygen consumption and free radical production localise in mitochondrial membranes. The sensitivity to oxidative damage of these membranes is strongly dependent on their unsaturated fatty acid content (polyunsaturated fatty acids), which are among the more susceptible cellular macromolecules to oxidative stress. At the same time, the lipid environment can directly affect membrane function, including mitochondrial electron transport and possibly oxygen free radical production [36]. In aerobic cells, active oxygen species, mainly superoxide and hydrogen peroxide, are generated as by-products of oxidative metabolism in mitochondria. These species are toxic to biomembranes and eventually lead to peroxidation of lipids unless they are removed by free radical-scavenging enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase. Antioxidants act to scavenge free radicals by converting them to less harmful molecules [6, 30]. Catalase (E.C.1.11.1.6), an iron-containing haemoprotein, converts hydrogen peroxide to water and oxygen [29]. A variety of natural antioxidants exist to scavenge oxygen free radicals and prevent oxidative damage to biological membranes. One group of these antioxidants is enzymatic (intracellular), which include superoxide dismutase, glutathione peroxidase and catalase. In addition to enzymatic antioxidants, the major natural antioxidants, most of them deriving from natural sources by dietary intake are vitamin A, vitamin C and vitamin E and carotenoids. Also, numerous small molecules are synthesized or produced within the body and have antioxidant capacity (e.g. glutathione and uric acid) [9, 12, 20].

The free radical genesis in virus infections is of great importance, because evidence suggests that nitric oxide and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases [3]. In farm animals, oxidative stress may be involved in several pathological conditions leading to economic losses and alteration of the animal welfare. Oxidative damage result from imbalance between oxidant production and neutralisation and induce oxidative modifications of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage [18]. Several studies showed that poxvirus leads to remote organ injury and that oxygen-based reactants may play a central role in this injury. Although no report showing a direct relation between Poxvirus and free radical accumulation was found, Poxvirus would cause severe damage, as indicated by free radicals and loss of membrane phospholipids [14].

The aim of the present study is to investigate the possible relationship between oxidative stress and clinical signs by evaluating the poxvirus effects on lipid peroxidation levels and antioxidant status in infected sheep.

Material and Methods

ANAMNESIS AND CASE REPORT

In a sheep herd of 400 Akkaraman sheep (from Kizilay district in Elazig, Turkey) the owner reported that 70 animals were ill for 4-5 days. All sheep of the herd were clinically examined. Sick animals were inactive, exhibited anorexia, coughing, lacrimation and nasal discharge. Specific poxvirus skin lesions (cf. results) were observed especially in unwooled areas. A single dose (10 mg/kg) of parenteral antibiotic (oxytetracyclin, Primamycin / LA, Pfizer) was intramuscularly injected to all sick sheep by the owner. The clinical examination has allowed to diagnose poxvirus infection and the Ministry of Agriculture was warned.

SAMPLE COLLECTION, HAEMATOLOGICAL AND BIOCHEMICAL INVESTIGATIONS

Blood samples were sampled from 20, 3-5 years old, diseased animals (test group) and 20 healthy 3-5 years old animals (control group) stemming from a not-infected herd by puncture of the jugular vein. A part of the blood was collected into sterile tubes containing EDTA (10 mL) for determination of haematological parameters, of malondialdehyde (MDA) and reduced glutathione (GSH) concentrations as well as catalase activity: after measurement of GSH concentrations, the EDTA-whole bloods were centrifuged at 1 500 g for 10 min at 4°C: plasmas were carefully harvested and kept at -20°C until MDA determination, whereas erythrocytes were washed three times with 0.9% NaCl to prepare erythrocyte haemolysates for measurement of catalase activities. Blood samples collected into sterile tubes without anticoagulant (20 mL) were allowed to clot at room temperature and were then centrifuged at 1 500 g for 10 min: the removed sera were kept at -20°C until determination of antioxidant vitamins (A, E, C and β-carotene).

Haematological parameters were determined by blood cell counter (Forcyte, USA).

Plasma MDA concentrations were measured by the modified method of SATOH [28] based on the reaction with thiobarbituric acid and were expressed as μmol/L. The CAT activities were determined by measuring the decomposition of hydrogen peroxide at 240 nm, according to the method of AEBI [1], and were expressed as expressed as 10^3 k/L. The GSH concentrations were measured by an assay using the dithionitrobenzoic acid recycling method described by ELLMAN [8] and were expressed as mmol/L. The determination of serum vitamin A and β-carotene concentrations was performed spectrophometrically according to the SUZUKI and KATOH's method [33]; the concentrations of vitamins C and E were determined according to the methods of KYAW [16] and MARTINEK [21] respectively.

STATISTICAL ANALYSIS

SSPS MS Windows Release 10.0 was used in order to determine significant differences between control and test groups with t-test. The difference was considered significant when p value was less than 0.05.

Clinical findings	Control group	Test group	p
Body temperature (°C)	39.16 ± 0.03 39.00 – 39.40	39.80 ± 0.11 (39.00 – 40.80)	< 0.001
Heart rate (/ min.)	71.2 ± 1.1 (60.0 – 76.0)	98.0 ± 3.3 (72.0 – 124.0)	< 0.001
Breathing rate (/ min.)	22.4 ± 0.4 (20.0 – 24.0)	27.0 ± 1.4 (20.0 – 44.0)	< 0.01
Rumen movement (/ 5 min.)	8.6 ± 0.1 (8.0 – 9.0)	2.6 ± 0.2 (0.0 – 4.0)	< 0.001

TABLE 1: Clinical findings (body temperature, heart and breathing rates, rumen movements) in 20 poxvirus infected sheep from the infected herd compared to healthy controls (n = 20) stemming from a not infected herd.
Results are expressed as mean ± standard deviation. Extreme values are given into parenthesis.

Haematological parameters	Control group	Test group	p
Total leukocyte count ($x10^9$/L)	7.67 ± 0.41 4.88 – 14.44	8.51 ± 0.59 (3.50 – 14.20)	NS
Erythrocyte count ($x10^{12}$/L)	8.40 ± 0.23 (7.17 – 10.73)	8.13 ± 0.20 (6.73 – 9.54)	NS
Packed cell volume (%)	27.9 ± 0.5 (24.3 – 30.2)	27.3 ± 0.5 (23.5 – 31.7)	NS
Haemoglobin (g/L)	92.2 ± 1.9 (80.0 – 106.0)	84.7 ± 2.1 (70.0 – 104.0)	< 0.05
MCV (fl)	33.2 ± 0.5 (28.8 – 39.0)	33.7 ± 0.6 (29.9 – 40.7)	NS
MCH (pg)	10.9 ± 0.1 (9.9 – 12.1)	10.5 ± 0.2 (8.8 – 12.0)	NS
MCHC (g/L)	329.8 ± 3.1 (290.0 – 344.0)	310.8 ± 4.6 (270.0 – 353.0)	< 0.01
Thrombocyte count ($x10^9$/L)	150.7 ± 10.6 (101.0 – 239.0)	89.2 ± 14.1 (11.0 – 198.0)	< 0.001

MCV: Mean Corpuscular Volume;
MCH: Mean Corpuscular Haemoglobin content; MCHC: Mean Corpuscular Haemoglobin Concentration

TABLE 2: Haematological parameters in 20 poxvirus infected sheep from the infected herd compared to healthy controls (n = 20) stemming from a not infected herd.
Results are expressed as mean ± standard deviation. Extreme values are given into parenthesis.

Results

CLINICAL FINDINGS

Papules and pustules were observed in the skin of all sick animals, especially on unwooled regions (udder, under tail, perianal area and inside surface of the legs). Granulomas were determined by palpation on the head of some sheep. All animals had serous, mucous or muco-purulent nasal discharge and almost all of them had lachrymal secretion. Some sheep exhibited coughing, a hard vesicular sound in the lungs and keratitis on one eye. Hypertrophy of superficial lymph nodules was sometimes observed.

Body temperature, heart and breathing rates and frequency of rumen movements were measured on the 20 selected diseased animals and on the 20 healthy controls (Table I). The body temperature was significantly elevated in the test group compared to the control group ($p < 0.001$) and 13 animals exhibited a body temperature above 39.5°C. In the same way, the heart and breathing rates were also markedly increased ($p < 0.001$ and $p < 0.01$, respectively). The heart rate was superior to 75 battements / minute in 19 animals and the respiratory frequency was above 25 cycles / minute in 9 animals. The rumen movements were dramatically depressed in the 20 diseased sheep (< 4 movements / 5 minutes) ($p < 0.001$).

HAEMATOLOGICAL FINDINGS

The White Blood Cell (WBC) count was slightly but not significantly increased in the test group (Table II). By contrast, thrombocyte counts were notably lowered ($p< 0.001$) compared to the controls: 9 animals presented thrombocyte counts below 100.10^9/L. Furthermore, the haemoglobinemia was also significantly diminished in the diseased animals ($p < 0.05$) and the erythrocytes were significantly less loaded with the blood pigment (significant decrease of the MCHC: $p < 0.01$).

Blood antioxidants / oxidants	Control group	Test group	p
MDA (mmol/L)	1.27 ± 0.21 (0.05 – 3.12)	5.37 ± 0.32 (3.43 – 9.98)	< 0.001
Catalase activity (10^3 k/L)	1.23 ± 0.09 (0.43 – 1.94)	1.54 ± 0.07 (0.55 – 2.11)	< 0.05
GSH (mmol/L)	5.9 ± 0.9 (1.2 – 12.5)	3.3 ± 0.5 (0.9 – 9.6)	< 0.05
Vitamin C (mg/L)	6.9 ± 0.6 (4.6 – 14.8)	5.3 ± 0.3 (1.7 – 8.3)	< 0.05
Vitamin E (mg/L)	2.1 ± 0.1 (1.5 – 4.0)	0.9 ± 1.6 (0.7 – 2.2)	< 0.001
Vitamin A (mg/L)	0.54 ± 0.06 (0.20 – 1.01)	0.49 ± 0.05 (0.22 – 1.0)	NS
β-carotene (mg/L)	0.05 ± 0.009 (0.03 – 0.17)	0.04 ± 0.004 (0.003 – 0.06)	NS

TABLE 3: Antioxidant / oxidant balance in 20 poxvirus infected sheep from the infected herd compared to healthy controls (n = 20) stemming from a not infected herd.
Results are expressed as mean ± standard deviation. Extreme values are given into parenthesis.

ANTI-OXIDANT STATUS

As shown in Table III, plasma MDA concentrations and catalase activities were markedly enhanced ($p < 0.001$ and $p < 0.05$, respectively) in affected group (test group) whereas blood GSH ($p < 0.05$), vitamin C ($p < 0.05$) and vitamin E ($p < 0.001$) concentrations were significantly depressed.

Slight decreases of serum vitamin A and β-carotene concentrations were also observed in the poxvirus infected sheep but the differences with controls were not statistically significant.

Discussion

Sheep poxvirus infection, a known most frequently occurred viral sheep disease, is observed frequently in Turkey and listed within the diseases group that have to be reported whenever observed, according to Health Protection Rule [4]. The clinical findings reported for this disease such as fever, depression, anorexia, conjunctivitis, lacrimation, rhinitis and difficulties in breathing, and then formation of papules, vesicles, pustules and scabs on the unwooled region of the skin [2, 15, 23, 24] and other findings such as skin thickening, lymphadenitis and pneumonia [15, 23] were also observed in the sick animals in the present study. Protective treatments against secondary infections were applied to the studied animals as described in literature [2, 5, 15, 22]. Although laboratory diagnosis could be possible via virus isolation and serological assays [15, 19, 27], severe clinical findings (widespread pox lesions in skin and mucosa) are indicated as pathognomonic.

Although body temperature ($p < 0.001$), heart ($p < 0.001$) and breathing ($p < 0.01$) frequencies were markedly elevated in the test group compared to the control group, the values were within the limits of usual values reported in the literature [32], probably because of the previous antibiotic treatment to animals made by the owner. The significant decreases in rumen movements ($p<0.001$) in the test group could be related to inappetence.

Haemoglobinemia ($p < 0.05$) and the haemoglobin load of erythrocyte (MCHC) ($p < 0.001$) were also significantly altered is the test group, albeit the observed values were included in the ranges of usual values reported in literature [15, 31, 32]. The decreases of these 2 parameters would be related to anaemia observed in sick sheep. Furthermore, a marked thrombocytopenia occurred in infected animals ($p < 0.001$ compared to controls). A dramatic decrease of the thrombocyte count was often described in viral, bacterial and protozoa infections [34].

The oxidant / antioxidant balance was notably affected in poxvirus infected sheep. The severe increases of plasma MDA concentrations observed in the test group compared to control ($p < 0.001$) indicate a high production of free radicals. As catalase is responsible for the O_2 formation from the hydrogen peroxide H_2O_2, the enzyme can be indirectly involved in the formation of radical oxygen species (ROS) [12, 17, 24, 37]. Consequently, it can be assumed that the poxvirus infection leads to an oxidative stress characterized by an important ROS production, evidenced by significant increases of plasma MDA concentrations and blood catalase activities. In parallel, blood concentrations of antioxidants (Vitamins E and C, GSH) were considerably reduced in the test group ($p < 0.001$ for vitamin E and $p < 0.05$ for GSH and vitamin C), suggesting a strong consumption of the antioxidants by the radical attacks. However, concentrations of vitamins C and E remained within usual values [26] even in diseased animals. As vitamin E acts as a potent scavenger of free radical in cell membranes [12, 17, 29, 35], the decrease of its serum concentration suggest that membrane lipid peroxidation occurs. Moreover, the reduction of vitamin C and GSH concentrations would be related to the alteration of vitamin E content in membranes; indeed, ascorbate is involved in reduction of tocopheryl radicals and vitamin E regeneration

in a non enzymatic reaction and in turn, ascorbate is regenerated by thiol transferase using the reduced glutathione (GSH) as substrate [9, 10, 30]. On the other hand, β-carotene concentrations were unexpectedly very low in both control and test groups, far below 20 mg/L as reported in healthy sheep [34]. These low values could be attributed to a weak β-carotene ingestion during winter when animals were not on the pasture [5] and to a rapid conversion of the pro-vitamin into vitamin A. Serum vitamin A concentrations in the both groups were within physiological values previously report for sheep [25, 26], but they tended to be weakly decreased in the test group, as well as β-carotene concentrations. The rapid mobilisation of liver vitamin A stores [4, 5, 32] has probably masked the antioxidant consumption by free radicals.

In this study, we have shown that poxvirus infection in sheep induced a marked oxidative stress evidencing by increases of plasma MDA concentrations and blood catalase activities, leading to severe consumption of antioxidants (mainly vitamins E and C and glutathione). Consequently, antioxidant (vitamins E, C and A at a lesser extent) administration may be useful as adjuvant treatment of sick sheep.

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