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Serum malondialdehyde, coenzyme Q10 and 8-hydroxy-2-deoxyguanosine levels in calves with foot-and-mouth disease

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Summary

Foot-and-mouth disease (FMD) is an acute, contagious viral disease in cattle that is associated with enormous economic losses in Turkey and worldwide. The purpose of this study was to determine changes in serum malondialdehyde (MDA), coenzyme Q10 (CoQ10), 8-hydroxy-2-deoxyguanosine (8-OHdG) and deoxyguanosine (dG) and to perform histopathological examinations in calves with FMD. Thirty calves were studied, 20 of which were infected with FMD and 10 were free of the disease. Following a routine clinical examination, blood samples were obtained, and serum MDA, CoQ10, 8-OHdG and dG levels were determined. Necropsy and histopathological examinations were performed on dead calves with FMD. MDA and 8-OHdG/10^6dG levels were significantly higher in calves with FMD than in the control group. However, the increase in CoQ10 levels in calves with FMD, compared with the control group, was not statistically significant. Macroscopic examination of the heart tissue of calves with acute myocarditis revealed the presence of pale, yellowish gray-white necrotic muscle fibers in the ventricular wall of the heart. The muscle fibers in the myocardium were swollen and exhibited pyknotic nuclei and intense lymphocytic cell infiltration. In longitudinal sections, the muscle fibers were non-striated, swollen, and homogenously pink and contained pink nuclei. Between muscle fibers, intense mononuclear cell infiltration was observed. The findings of the present study indicate that oxidative stress is significantly increased in calves with FMD, and that oxidative DNA damage may play an important role in the etiopathogenesis of FMD. This is the first study to report CoQ10 and 8-OHdG levels in calves with FMD, and its findings may serve as the basis for future studies on this subject.

Keywords: foot-and-mouth disease, myocarditis, MDA, CoQ10, 8-OHdG

Foot-and-mouth disease (FMD) is an acute, contagious viral disease that affects domestic and wild cloven-hoofed animals and leads to enormous economic losses (23, 27). It is caused by aphthovirus, an RNA virus (FMDV) from the family Picornaviridae (1). There are seven serotypes of FMDV, namely O, A, C, SAT 1-3 and Asia 1, and about 60 subtypes. FMD occurs enzootically in Africa, Asia (including Turkey) and occasionally in South America (1, 6, 8, 12, 27).

FMD is common in domestic animals, such as cattle, sheep, goats and swine, but the disease progresses more severe in cattle than it does in sheep or goats (1, 8, 23). The clinical signs of this disease in cattle are increased body temperature, loss of appetite, difficulty in eating, vesicle formation in the mouth, on the tongue, gums, palate, udder and coronary band, as well as lameness, and the drooling of stringy saliva. The clinical course worsens as a result of secondary infections. The virus has an affinity for the cardiac muscle, and macroscopic observation reveals a tiger fur-like appearance of the heart in animals with affected cardiac muscles (1, 8, 14). This affinity for the cardiac muscle makes FMD particularly devastating in young animals, which eventually die as a result of muscle damage and myocarditis

(14, 27). Therefore, while the mortality rate is 5% in adult animals, it may reach up to 50% in young animals (1, 8, 14, 23).

In order to obtain detailed information about diseases in animals, it is important to measure biochemical parameters and compare them between infected animals and healthy animals (10). Changes in various biochemical parameters, especially those related to cardiac function, have been reported in animals with FMD, especially young ones. However, it is important to explore other biochemical markers in order to improve the diagnosis and treatment of this disease (6, 12, 23).

Occasionally, the balance between oxidants and antioxidants may be altered in certain conditions, and as a result, oxidative stress may occur. In normal conditions, free radicals are produced during cellular metabolism. When antioxidants are absent or few, these free radicals may have an effect on lipids, carbohydrates, proteins and nucleic acid, and thereby lead to oxidative stress (3, 19, 21). Oxidative damage caused by oxidative stress is believed to play a role in the pathogenesis of several diseases, including cardiovascular disorders (19, 21). There are several biochemical markers of oxidative stress, including malondialdehyde (MDA). MDA is the final product of lipid peroxidation, and it is therefore commonly used as an indicator of lipid peroxidation. In addition, it has adverse effects on ion transport, enzymatic activities and cell membranes, and it is used as an indicator of the severity of cellular damage (3, 7).

Another marker of oxidative stress is ubiquinone 10 (CoQ10), which is a lipid structured compound of the mammalian mitochondrial electron chain that prevents damage caused by biomolecules and lipid peroxidation by playing a role in the production of active forms of ascorbic acid and tocopherol (9, 16). CoQ10 is important for cardiac muscle function, and it plays a role as an antioxidant in cardiac disorders and heart failures. CoQ10 is a strong antioxidant that protects cellular structure by eliminating free radicals (9, 16, 18).

8-hydroxy-2-deoxyguanosine (8-OHdG) is the most abundant marker of oxidative DNA damage. Reactive oxygen species (ROS) are produced when antioxidants are absent or scarce. These ROS may damage lipids, proteins and particularly DNA (4, 5, 20, 22). When DNA suffers oxidative damage as a result of ROS, 8-OHdG is produced as one of the products of the damage. 8-OHdG is one of the best characterized DNA markers associated with cellular damage. 8-OHdG is an indicator of aging, carcinogenesis and oxidative stress in animals and humans that can be measured in samples, such as urine, tissue, leucocyte DNA and even serum (3, 19, 21, 26). While numerous studies have evaluated these parameters of oxidative stress in human diseases (5, 9, 16, 24), there are no studies on serum MDA, CoQ10 and 8-OHdG levels in calves with FMD. In addition, in most biochemical studies on FMD in calves, mainly parameters related to cardiac markers such as troponin, troponin T, troponin I etc. were examined (6, 12, 23).

The purpose of the study was to assess changes in serum MDA, CoQ10, 8-OHdG and deoxyguanosine (dG) levels and to examine the histopathological features of calves with FMD.

Material and methods

The study included 30 calves, 20 of which had FMD and 10 were healthy. The calves were of different breeds (Holstein and Simmental), ages (1-6 months) and sexes. The animals were brought to our clinics from the cities of Van and Erzurum. Blood samples from the jugular vein were collected into tubes containing EDTA and tubes without EDTA. Blood samples in anticoagulant-free tubes were centrifuged (Rotofix 32®-Hettich) for 10 min at 3000 rpm, and serum samples were extracted. MDA, CoQ10, 8-OHdG and dG levels were measured at Van Yuzuncu Yil University, Faculty of Medicine, Department of Biochemistry, with a high-pressure liquid chromatography (HLPC) device (Agilent 1200 modular system, Boblingen, Germany).

To obtain definite diagnosis and to identify serotypes, the blood samples were delivered to the Ankara Foot-and-Mouth Disease Institute via the Provincial Directorate of Food, Agriculture and Livestock. According to the results of the Foot-and-Mouth Disease Institute, there were four ASIA 1 and sixteen A serotypes in the samples. Blood samples positive for FMD were used in this study.

Calves that died despite treatment were delivered to the pathology laboratory for pathological and histopathological examinations and systemic necropsy. Pathological findings were recorded, and in order to perform histological examinations, heart tissues with lesions were fixed in a 10% formalin solution for 48 h and washed under flowing tap water for 10 h. For the histological examination, tissues were embedded in paraffin blocks after they had been passed through an alcohol and xylol series. Sections of 4 μm thickness were obtained from every block, and the tissues were prepared on microscope slides. The prepared tissues were stained with hematoxylin-eosin (H&E) and examined under a light microscope (Leica DM 1000, Germany).

MDA analysis. The MDA concentration in the serum samples was measured with an HPLC device (Agilent 1200 mobile system; Boblingen, Germany) by a previously described method (13). For HPLC, 750 μL H_3PO_4 (0.44 mol/L) and 250 μL thiobarbutiric acid (TBA, 42 mmol/L) were added to 50 μL of the serum sample, and the solution was placed in a boiling water bath for 30 min. After cooling in icy water, alkaline methanol (methanol containing 4.5 mL of 1 M NaOH: total volume 54,5 mL) was added in equal volume to the samples. After strong vortexing, the samples were centrifuged at 3000 rpm for 3 min. The upper phase that formed was transferred to vials and placed in an HPLC device (HP, Agilent 1200 modulary system; Bonlingen, Germany). RP-C18 (particle size: 5 μm, length: 150 mm, diameter: 4.6 mm) columns were used. The mobile phase was an absolute methanol/50 mM KH_2PO_4 buffer of pH 6.8 (40 : 60, v/v). The flow rate and injection volume were adjusted to 0.8 μL and 20 μL, respectively. MDA measure-

ment was performed with a fluorescent detector at an excitation wavelength of 527 nm and an emission wavelength of 551 nm. The spike of the MDA-TBA complex was calibrated with a 1,1,3,3-tetraethoxypropane standard solution. MDA concentrations were expressed in μM.

CoQ10 analysis. The analysis of oxidated CoQ10 was performed according to the method used by Litarru et al. (15). This method involves a direct injection of 1-propanol into the HPLC device and forcing reduced CoQ10 to oxidize that exists in para-benzoquinon applied samples. For HPLC analysis, a reverse-phase ODS supercoil C-18 (particle size: 3 μm, length: 15 cm, inner diameter: 0.46 cm) was used as the column. Serum samples (200 μL) were mixed with 50 μL 1,4-benzoquinon (2 mg/mL) by vortexing. The solution was incubated at room temperature for 10 min, after which 1 mL n-propanol was added, and it was vortexed for 10 s. The mixture was centrifuged for 2 min at 600 rpm. Then, 200 μL of the supernatant was collected, transferred into a vial and loaded onto the HPLC device. For spectral analysis, the UV detector was adjusted to 275 nm, and the flow rate of the ethanol/methanol (65 : 35, v/v) mobile phase was adjusted to 1 mL/min. When the column was stabilized, oxidized CoQ10 was measured with the UV detector. The findings are shown in μM.

8-OHdG and dG measurement. In order to extract DNA from leukocytes separated from whole blood, a DNA isolation kit (GenAll DNA extraction kit; GenAll Biotechnology Co.Ltd., Seoul, Korea) including the spin column method was used according to the manufacturer's instructions The isolated DNA samples were hydrolyzed with formic acid at 150°C for 8-OHdG analysis according to a previously described method (11). The hydrolyzed DNA samples were dissolved in acetonitrile (final volume, 1 mL), and 8-OHdG and dG levels were measured with ECD and UV detectors, respectively, in an HPLC device. A reverse-phase C-18 (RP-C18) analytical column (particle size: 4.0 μm, diameter: 250 mm, length: 4.6 mm; Phenomenex, CA) was used for HPLC. The mobile phase was a 0.05 M potassium phosphate buffer (pH 5.5) and absolute acetonitrile mixture (97 : 3, v/v). The flow rate was adjusted to 1 mL/min, and injection volume was adjusted to 20 μL. In the HPLC device, the amount of 8-OHdG was determined by adjusting the ECD detector to 600 mV, and dG was measured at an absorbance wavelength of 245 nm. The 8-OHdG and dG standards used for 8-OHdG and dG measurement were provided by Sigma Aldrich. The 8-OHdG values were expressed as 8-OHdG/10^6dG for each 10^6dG (22).

Histopathological examinations. Systemic necropsy was performed on dead calves. For histopathological evaluation after necropsy, heart muscle tissues obtained from the bodies were fixed in a 10% formalin solution for 48 h and washed under tap water for 10 h. For routine tissue tracking, the samples were passed through an alcohol and xylol series prior to embedding in paraffin blocks. Sections of 4 μm thickness were obtained from every block and prepared on microscope slides. The preparations were stained with H&E and examined under a light microscope.

Statistical analyses. For continuous variables, definitive statistical values were expressed as mean and standard deviation. Following variant analysis, Student's t test was used for comparisons between groups. Statistical significance was accepted at 5% ($p < 0.05$), and the SPSS 20 software (SPSS Inc., IL, USA) was used for calculations.

Results and discussion

Clinical symptoms. According to the anamnesis of the animal owners, the clinical signs in the adult animals were similar to those for FMD. However, some of the calves that were found dead had not exhibited any clinical signs. A routine clinical examination was performed on calves brought to our clinics. Physical examination revealed increased body temperature and the presence of vesicles in the mouth, on the tongue and gums. Further, erosions and ulcer formation were observed in these regions after rupture of the vesicles, as well as lameness in some animals. In some calves, none of the classic symptoms of FMD were found. Increased body temperature, heart rate and respiratory rate were noted in all animals.

Values of biochemical parameters. The oxidative stress parameters assessed are shown in Tab. 1 and Fig. 1. The levels of 8-OHdG/10^6dG and MDA were significantly higher in calves with FMD than in the control groups (MDA: 2.541 ± 0.219 μM vs. 1.962 ± 0.126 μM, 8-OHdG/10^6dG: 1.481 ± 0.156 vs. 1.028 ± 0.146; $p < 0.05$ for both). Oxidized CoQ10 levels were higher in the calves with FMD, but the difference in comparison with the control group was not statisti-

Tab. 1. Values of selected oxidative stress parameters in control group and in calves with foot-and-mouth disease

Parameter	Control Group n = 10	Calves with FMD n = 20
MDA (μM)	1.962 ± 0.126	2.541 ± 0.219*
CoQ10 (μM)	1.495 ± 0.144	1.719 ± 0.251
8-OHdG/10^6dG ratio	1.028 ± 0.146	1.481 ± 0.156*

Explanation: * – significant compared to control group calves

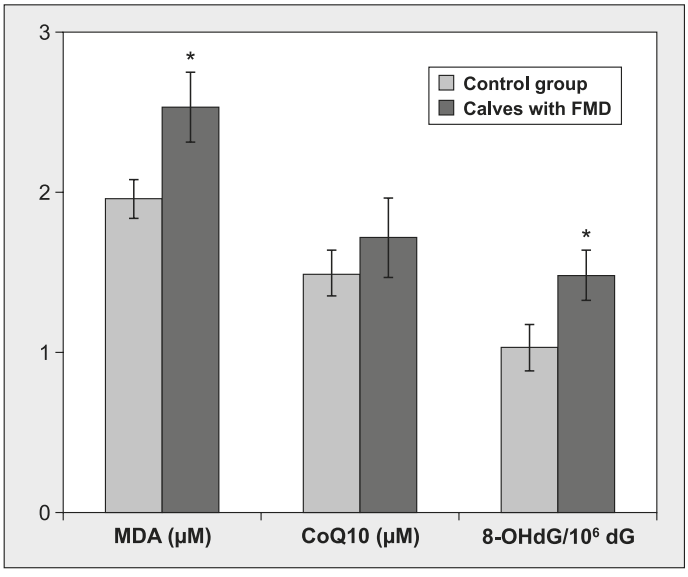

Fig. 1. Comparison of oxidative stress parameters in control group and in calves with foot-and-mouth disease

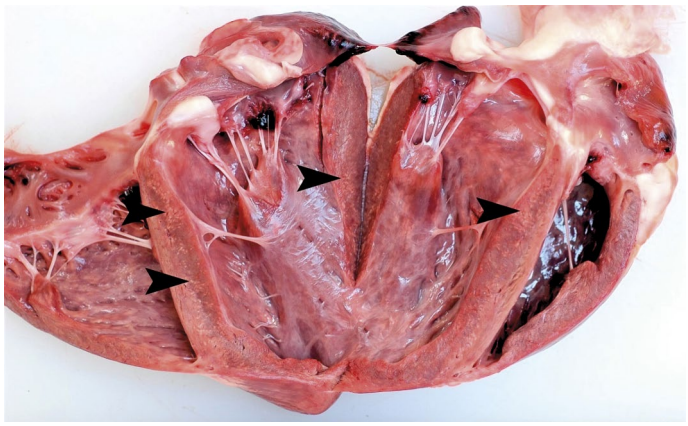

Fig. 2. Acute necrotic myocarditis in heart muscle (arrow heads)

cally significant (1.719 ± 0.251 µM vs. 1.495 ± 0.144 µM, $p > 0.05$).

Macroscopic and microscopic changes. Calves with acute myocarditis exhibited pale, yellowish gray-white necrotic muscle fibers in the ventricle wall of the heart (Fig. 2). In calves that died of acute myocarditis, coagulated blood was observed in the ventricle lumen, as well as pulmonary edema and a small amount of exudate in the pericardium and in the thoracic and abdominal cavities.

In cardiac tissue, myocardial muscle fibers were swollen, and the nuclei were pyknotic; further, intense lymphocytic cell infiltration was observed in this region (Fig. 3). In longitudinal sections, muscle fibers were non-striated, swollen and homogenously pink and had pyknotic nuclei. Further, intense mononuclear cell infiltration was observed between muscle fibers (Fig. 4). This cellular infiltration was so intense in certain regions that muscle fibers there could not be clearly observed (Fig. 5).

The clinical examinations conducted in this study showed an increase in body temperature, presence of vesicles in the mouth, on the tongue and gums, as well as erosions and ulcerations in places where the vesicles had ruptured. Lameness was also observed in some calves. These clinical findings are similar to those reported in previous studies on FMD (1, 8, 14). Some of the calves included in the present study died suddenly before exhibiting any clinical symptoms. We believe that the reason for the sudden death was acute myocarditis. This is corroborated by other studies (14, 27), which report that in young animals this disease may lead to death caused by myocarditis, since the virus has affinity for cardiac muscle.

In this study, coagulated blood was observed in the ventricle lumen of the heart, as well as pulmonary edema and a small amount of exudate in the pericardium and in the thoracic and abdominal cavities. In addition, pale, yellowish gray-white necrotic muscle fibers were observed in the ventricle wall muscle. Microscopic observation of the cardiac tissue revealed that the muscle fibers in the myocardium were swollen

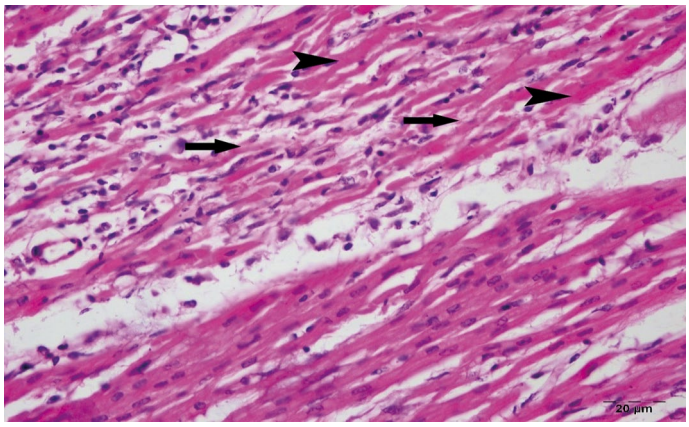

Fig. 4. Longitudinal section of cardiac muscle: hyalin degeneration in muscle fibers (arrows), Zenker necrosis (arrow head), mononuclear cell infiltration between muscle fibers, H&E Bar: 20 µm

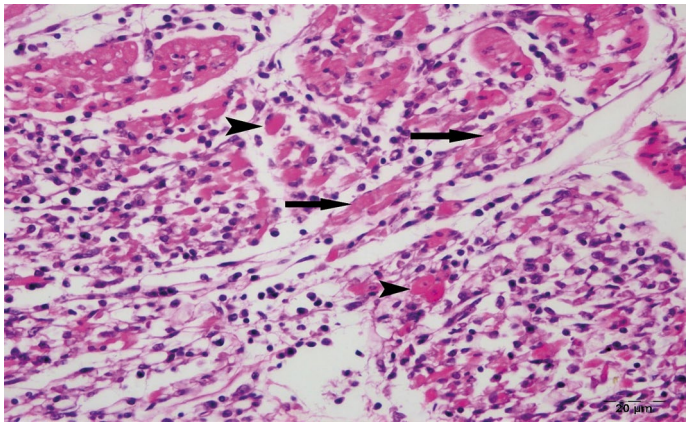

Fig. 3. Transverse section of cardiac muscle: hyalin degeneration in muscle fibers (region shown with arrows), Zenker necrosis (arrow head), intense mononuclear cell infiltration, H&E Bar: 50 µm

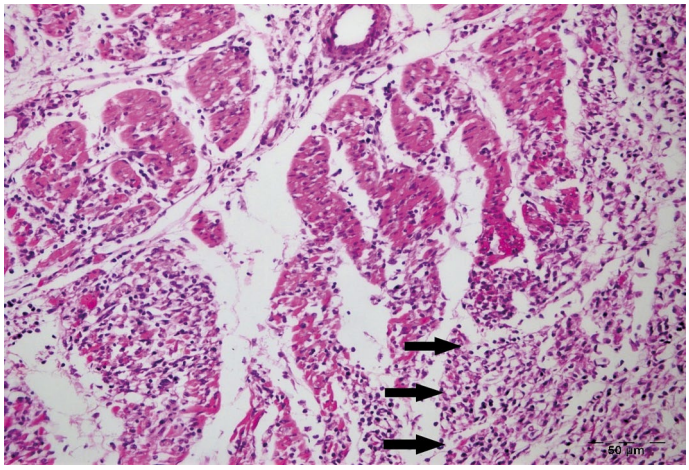

Fig. 5. Transverse section of cardiac muscle: hyalin degeneration in muscle fibers, Zenker necrosis, intense mononuclear cell infiltration (region shown with arrows), H&E Bar: 50 µm

and had pyknotic nuclei, and an intense lymphocytic cell infiltration occured in these regions. In longitudinal sections, muscle fibers were non-striated, swollen and homogenously pink and had pyknotic nuclei, and an intense mononuclear cell infiltration was observed

between muscle fibers. The cellular infiltration was too intense for a clear observation of muscle fibers in certain regions. These macroscopic and microscopic findings are similar to previously reported findings (1, 8, 12).

Oxidative stress plays an important role in several pathological events, including the pathogenesis of FMD (2, 17, 25). It has been reported that while the total oxidant capacity is increased in cattle with FMD, the total antioxidant capacity remains unchanged (2). Another study (25) reported that serum and saliva MDA levels were significantly higher in bulls with FMD compared to control animals, and that GSH levels decreased dramatically as antioxidant capacity weakened. Yet another study reported that MDA levels were significantly increased in animals with FMD, and that antioxidant treatment should be administered in viral diseases, such as FMD (17). In addition, increased MDA parameters and decreased SOD and total antioxidant capacity have also been reported in FMD (27). Similar to these published reports, in this study, MDA levels and the 8-OHdG/10^6dG ratio in calves with FMD were significantly higher than those in the control group. However, although oxidized CoQ10 levels were higher in calves with FMD, the difference was not significant compared to the control group. None of the studies on FMD in calves has examined CoQ10 and 8-OHdG levels, which makes our study unique in its contribution to the biochemical study of FMD.

The findings of the present study indicate that oxidative stress was significantly increased in calves with FMD. This means that lipid peroxidation and oxidative DNA damage may play an important role in the etiopathogenesis of FMD. This is the first study to report CoQ10 and 8-OHdG levels in calves with FMD, and its findings will form the basis for further studies on the role of oxidative stress in FMD.

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