RESEARCH ARTICLE

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Evaluation of 4-Hydroxy-2-Nonenal, Dityrosine and 8-Hydroxy-2-Deoxyguanosine Expressions in Lambs with White Muscle Disease

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The aim of this study was to investigate Reactive Oxygen Species (ROS)-induced lipid peroxidation, protein and DNA damage with oxidative stress markers such as 4-hydroxy-2-nonenal (4-HNE), dityrosine (DT) and 8-hydroxy-2-deoxyguanosine (8-OHdG) in lambs with White Muscle Disease (WMD). The material of this study consisted of tissue samples taken from 20 sheep with WMD and 6 healthy control groups brought to Pathology Department for routine histopathological diagnosis between 2012 and 2019. In macroscopic examinations, chalky-white large necrotic areas on the epicardial and especially on the endocardial surfaces of the right and left ventricular walls were observed. In microscopic examinations, it was observed that the degenerated muscle fibers in the heart were homogeneously pink in color, lost their cytoplasmic citration, swelled and their nuclei were pyknotic. Calcifications in necrotic and degenerated areas were detected. All white muscle disease cases were immune positive for 4-HNE, DT and 8-OHdG expressions in heart tissues. A significant difference ($P<0.05$) was detected between the groups in terms of immunoreactivity to 4-HNE, DT and 8-OHdG in immunohistochemical and immunofluorescence staining findings. In conclusion, our study showed that formation of ROS that is important in pathogenesis of WMD causes not only lipid peroxidation, but also protein modification and DNA damage.

Key Words: *4-HNE, 8-OHdG, dityrosine, lambs, white muscle disease*

Beyaz Kas Hastalıklı Kuzularda 4-Hidroksi-2-Nonenal, Ditirozin and 8-Hidroksi-2-Deoksiguanozin Ekspresyonlarının Değerlendirilmesi

Bu çalışmada, beyaz kas hastalıklı kuzularda 4-hidroksi-2-nonenal (4-HNE), ditirozin (DT) ve 8-hidroksi-2-deoksiguanozin (8-OHdG) gibi oksidatif stres belirteçleri ile Reaktif Oksijen Türleri (ROT) kaynaklı lipid peroksidasyonu, protein ve DNA hasarının araştırılması amaçlanmıştır. Bu çalışmanın materyalini 2012-2019 yılları arasında rutin histopatolojik tanı için Patoloji Anabilim Dalı'na getirilen beyaz kas hastalıklı 20 koyundan alınan doku örnekleri ve 6 sağlıklı kontrol grubu oluşturdu. 4-HNE, DT ve 8-OHdG ekspresyonları immünohistokimyasal ve immünofloresan yöntemlerle değerlendirildi. Makroskopik incelemelerde epikardiyal ve özellikle endokardiyal yüzeylerde, sağ ve sol ventrikül duvarlarında tebeşirimsi beyaz büyük nekrotik alanlar gözlemlendi. Mikroskobik incelemelerde kalpteki dejenere olmuş kas liflerinin homojen pembe renkte olduğunu, sitoplazmik sitrifikasyonunu kaybettiğini, şiştiği ve çekirdeklerinin piknotik olduğu gözlemlendi. Nekrotik ve dejenere alanlarda kalsifikasyonlar tespit edildi. Tüm beyaz kas hastalığı vakaları kalp dokularında 4-HNE, DT ve 8-OHdG ekspresyonu yönünden immün pozitifti. İmmünohistokimyasal ve immünofloresan boyama sonuçlarında 4-HNE, DT ve 8-OHdG'ye karşı immünreaktivite açısından gruplar arasında anlamlı farklılık ($P<0.05$) tespit edildi. Sonuç olarak çalışmamız beyaz kas hastalığının patogenezinde önemli bir faktör olan ROS oluşumunun hem lipid peroksidasyonuna, hem protein modifikasyonuna hem de DNA hasarına neden olduğunu göstermektedir.

Anahtar Kelimeler*: 4-HNE, 8-OHdG, ditirozin, kuzu, beyaz kas hastalığı*

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Introduction

White muscle disease (WMD), also known as subacute enzootic muscular dystrophy or stiff-lamb disease, is caused by Selenium (Se) and/or vitamin E (Vit E) deficiency. It is an important nutritional disease that is seen in our country as well as all over the world (1-3). The disease, has been reported especially in Central, Eastern and Southeastern Anatolia regions (4, 5). It affects not only sheep but also many domestic and wild animals such as goats, cattle, deer, horses, pigs, rabbits, marsupials, monkeys, laboratory and exotic animals and even fish (6). WMD is seen in young sheep grazing (stubble or rank) or being maintained on hay and grain rations (7). Disease is more common in lambs up to 3 months of age, it can also be seen in newborn lambs (2). The main clinical symptoms included weakness, unwillingness to move, difficulty in standing, stiff gait, curvature of the back, short and upright steps (8-10). Both Vit E and Selenium play important roles in protecting cellular membranes to the free radical-induced lipid peroxidation (9, 11). Se and Vit E deficiencies cause lipoperoxidation in tissues to muscle degeneration and calcification (3, 8, 12). Free radicals, which are formed as a result of the decrease in antioxidant defense due to Se and Vit E deficiency, have an important role in the pathogenesis of this disease and causing oxidative damage by causing toxic effects on enzymes, nucleic acids, polysaccharides and unsaturated fatty acids in cell membranes (5, 13, 14).

Elevated levels of reactive oxygen species (ROS) act as a marker for oxidative stress and they are associated with lipid peroxidation and imbalance of the redox system (15). Among the many aldehydes produced from lipid peroxidation is the α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (4-HNE), especially 4-HNE is the most studied lipid peroxidation end product (16, 17). 4-HNE, which is a specific marker of oxidative stress, has important electrophilic properties and reacts with many classes of biomolecules such as phospholipids, proteins and nucleotides, forming covalent adducts (18, 19). 3,3′-dityrosine, also known as dityrosine (DT), a stable and convenient biomarker for oxidative modifications of proteins, is generated by reactive species, attacking tyrosine residues in proteins and, eventually, generating tyrosyl radicals (20-22). Two tyrosyl radicals can form inter-molecular cross-links producing a dimer called DT (23). The hydroxyl radical attacks DNA strands when it is produced adjacent to cellular and mitochondrial DNA causing the addition of DNA bases radicals, which lead to generation of a variety of oxidation products, one of these oxidative DNA products, 8-hydroxy-2-deoxyguanosine (8-OHdG), is a biomarker that is extensively used to reflect the degree of oxidative DNA damage (24-26).

The aim of this study was to investigate ROS-induced lipid peroxidation, protein and DNA damage with oxidative stress markers such as 4-HNE, DT and 8-OHdG in lambs with WMD.

Material and Methods

Research and Publication Ethics: The ethics committee report of this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (Authorization number: KAU-HADYEK-2020/093).

Animals: The material of this study consisted of tissue samples (heart and gluteal muscle) taken from 20 sheep with WMD and 6 healthy control groups brought to our department for routine histopathological diagnosis between 2012-2019 years.

Histopathological Investigations: Tissue samples (heart and gluteal muscle) from lambs were fixed in 10% buffered formaldehyde solution, processed routinely, embedded in paraffin and sectioned at 5 µm and stained with Hematoxylin & Eosine (H&E). Sections were examined under a light microscope (Olympus Bx53) and photographed using the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4).

Immunohistochemical and Immunfluorescence Investigations: Paraffine sections of on Poly-L-lysine slides passed through xylol and alcohol series. After washing with PBS, sections were incubated for 10 minutes in 3% H_2O_2 for inactivation of endogenous peroxidase activity. In order to reveal the antigen in the tissues, the antigen was treated with retrieval solution at 500 watts for 2x5 minutes. The tissues were then washed with PBS and left for incubation for 0 minutes at room temperature with primary antibodies, 4-HNE (Abcam, Catalog no: ab 46545, anti rabbit, polyclonal), 8-OHdG (Santa Cruz, Catalog no: sc 66036, anti mouse, monoclonal) and Anti 3,3'Dityrosine (Biomol, Catalog no: MDT-020P). Secondarily; Mouse and Rabbit Specific HRP/DAB IHC Detection Kit - Micro-polymer kit (Abcam, Catalog No. ab236466, anti mouse, monoclonal) was used as recommended by the manufacturer. DAB (3,3′-Diaminobenzidine) was used as chromogen. After contrast painting with Mayer's Hematoxylin, it was covered with entellan and examined under a light microscope. In the immunohistochemical examination, findings ere classified as no immunoreactivity (-), mild (+), moderate (++) and severe (+++). In the immunofluorescence method, 45 min. Goat Anti-Mouse IgG H & L-FITC (cat. no. 6785, dilution 1/50, Abcam, UK) and Mouse Anti-Rabbit IgG H & L-FITC (cat. no. sc-2359, dilution 1/50, Santa cruz) secondary antibodies were applied. At the end of the incubation period, the washed sections were covered with 4’,6-diamidino-2-phenylindole (DAPI) fluorescence medium. Fluorescence was evaluated as absent (-), mild (+), moderate (++) and severe (+++) under the microscope.

Statistical Analysis: The data obtained were analyzed with the SPSS 20.00 program. The difference between the groups was determined by Mann Whitney U test, one of the nonparametric tests. A value of $P<0.05$ was considered statistically significant (27).

Results

Clinical Results: 18 of the 20 animals had an average age of 45 days. The remaining 2 animals were 10 days old. According to the anamnesis obtained from the animal owners, the animals had symptoms including anorexia, inability to get up from the ground, reluctance to move, hunched back, stiff gait, curvature of the back, short and upright steps, shortness of breath, inability to lift the head and limpness.

Macroscopic Results: In macroscopic examinations, pale, chalky-white large necrotic areas on the epicardial and especially on the endocardial surfaces, right (18/20, %90) and left (2/20, %10) ventricular walls were detected (Figure 1a). Necrotic changes were generally present in the heart muscle, however both the heart and gluteal muscles were affected in some cases. The gluteal muscles were typically looked like chicken meat (Figure 1b).

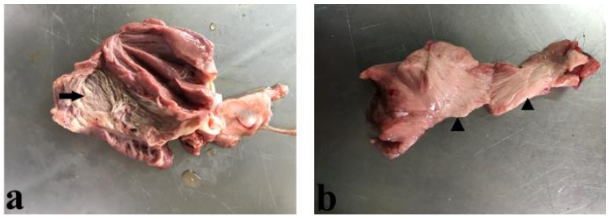

Figure 1. a: Chalky-white-looking Zenker’s necrosis (arrow) on the endocardial surface of the heart, **b:** Chicken meat appearance in the gluteal muscles (arrow heads)

Microscopic Results: Microscopic examinations showed that the degenerated muscle fibers in heart were homogeneously pink in color, lost their cytoplasmic citriation, were swollen, and their nuclei were pyknotic. We detected calcifications in necrotic and degenerated areas (Figure 2 a-b). Fibrosis was prevalent in some areas. Mononuclear cell infiltrates in the interstitial regions were among the other histopathological findings observed. The presence of hyaline degeneration and calcified areas in the gluteal muscles, in line with the findings in the heart muscle, was remarkable (Figure 2 c-d).

Immunohistochemical and Immunfluorescence Results: A significant difference was detected between the groups in terms of immunoreactivity to 4-HNE, Anti 3,3 'Dityrosine and 8-OHdG in immunohistochemical and immunofluorescence staining findings (Table 1, P<0.05). Immunoreactivity for 4-HNE, Anti 3,3 'Dityrosine and 8-OHdG could not be detected at a significant level in the heart muscle tissues of the control group animals. While the 8-OHdG and 4-HNE immunoreactivity of the animals in the WMD group was found to be mild in the heart muscle, the immunoreactivity for Anti 3,3 'Dityrosine was found to be at a severe level (Fig 3-4 a-c).

Table 1. 4-HNE, 8-OHdG and DT immunoreactivities of control and WMD groups

Groups	4-HNE	8-OhDG	Dityrosine
Control	0.33± 0.51[a]	0.16± 0.40[a]	0.33± 0.51[a]
WMD	1.33± 0.51[b]	1.16± 0.98[b]	2.83± 0.40[b]

[a, b] : Shows the difference in the same column (P<0.05)

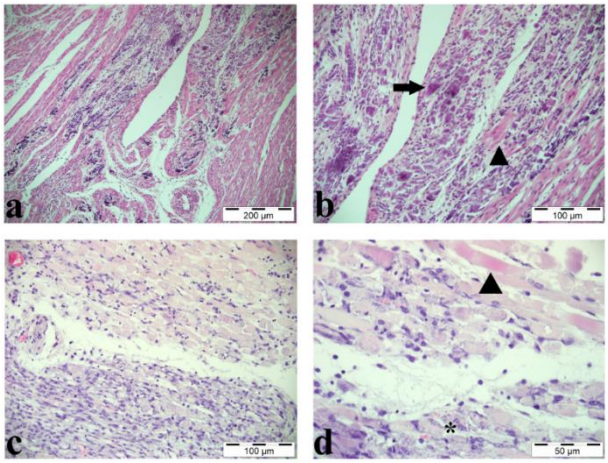

Figure 2. a-b: Heart muscle, different magnifications, calcification (arrow) and hyaline degeneration (arrowhead), H&E, **c-d:** Gluteal muscle, different magnifications, calcification (*) and hyaline degeneration (arrowhead), H&E

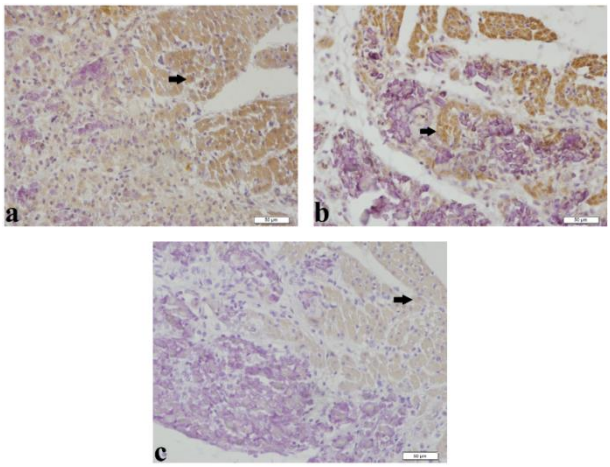

Figure 3. a-c: 4-HNE, 8-OHdG mild immunoreactivity in heart muscle (arrow), **b:** Severe immunoreactivity to DT (arrow), IHC, Bar= 50 µm

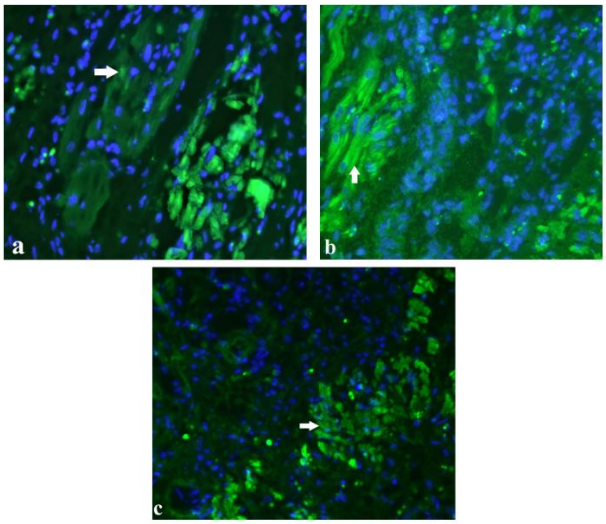

Figure 4. a-c: 4-HNE, 8-OHdG mild immunoreactivity in heart muscle (arrow), **b:** Severe immunoreactivity to DT (arrow), IF, 20X Magnification

Discussion

WMD is seen as congenital form in newborns or adoptive form in 3-4 months old lambs and the clinical course of the disease varies as peracute, acute and subacute forms (1, 6). The main clinical findings of the disease are as follows; inability to stand up, stiff-legged gait, shortness of breath, loss of appetite, weakness, unwillingness to move, difficulty in standing, stiff gait, curvature of the back, short and upright steps (4, 8). In our study, in accordance with the literature data (1, 6), the disease was detected mostly in animals up to 3 months (18 out of 20 animals, 90%). The remaining 2 animals were 1 week - 10 days old. In addition, many clinical symptoms such as anorexia, inability to get up from the ground, reluctance to move, hunched back, stiff gait, curvature of the back, short and upright steps, shortness of breath, inability to lift the head and limpness were noted in animals, as previously reported (4, 8, 28,

29). In the present study, the diagnosis of WMD in lambs was made based on observed macroscopic and microscopic lesions (6, 28). The gross (2, 7, 13) and histopathological lesions (29-31) in the heart and gluteal muscles we detected in lambs were similar to those previously reported by different researchers.

The most important factors in the etiology of WMD in lambs are Vit E and / or Se deficiency (5, 7). Se enters the structure of the enzyme of glutathione peroxidase (GSH-Px) which works in the body's antioxidant protection and reduces hydrogen peroxide, super-oxide radicals and lipid peroxides to water (4, 8). Vit E is a lipophilic antioxidant that reduces hydroperoxide formation and acts to scavenge free radicals at the extracellular or intracellular level (9, 14). Vit E and Se-containing GSH-Px, are an important part of the antioxidant system found in all cells. In WMD, lipid peroxidation and hydrogen peroxide normally occurring in the organism cannot be scavenged from the muscles due to the decrease in GSH-Px activity caused by Se and antioxidant Vitamin E deficiencies (11). Free oxygen radicals that develop due to the decrease in antioxidant activity play a serious role in the pathogenesis of the disease by causing severe pathological changes such as lipid peroxidation in tissues, degradation of proteins, degeneration and finally necrosis of the myocardium (3, 13). Therefore, an increase in oxidative stress and 4-HNE expression, which is an important active lipid peroxidation marker in heart tissue, is thought to be due to the decrease in antioxidant defense (17). As we expected, we found that 4-HNE expression increased statistically in the WMD group compared to the control group. In human medicine, there are findings that 4-HNE is produced in the heart in cardiovascular diseases such as atherosclerosis, hypertrophy, cardiomyopathy, myocardial ischemia-reperfusion injury and arrhythmias compared to healthy individuals in the control group of 4-HNE (32, 33). In the literature review, no study was found in which lipid peroxidation in lambs with WMD was evaluated in terms of 4-HNE expressions by immunohistochemical methods. Consistent with our results, different researchers found that lipid peroxidation increased in WMD animals compared to healthy animals in terms of Malondialdehyde (MDA) levels (1, 5, 14). DT is a prominent marker for oxidative stress as protein oxidation and is formed by ROS attack on a wide range of proteins (20, 22, 23). DT is an irreversible and irreparable oxidative modification (21). We did not find any literature data in which DT expressions were evaluated by immunohistochemical methods in white muscle disease. However, it has been reported that DT levels increase statistically in disease group compared to the control group for chronic heart failure and acute myocardial infarction models (22, 23, 33). In the present study, we observed that DT expression in the heart of the animals in the WMD group was quite severe and increased significantly compared to the control group. We interpreted this increase in DT immunoreactivity as ROS-induced protein modifications may play an important role in the pathogenesis of WMD. ROS can cause specific oxidative DNA damage and 8-OHdG is the most used biomarker to detect this damage (1, 24). 8-OHdG values have been evaluated in detail in human cardiovascular diseases such as coronary artery disease, non coronary artery disease, myocardial infarction, ischemic stroke, heart failure, atherosclerosis (25, 26). In the literature reviews, it is seen that there is a positive relationship between 8-OHdG levels and cardiovascular diseases (25, 26). However, there is only one study in which 8-OHdG levels were evaluated in immunohistochemical methods in lambs with WMD (1). We determined that the 8-OHdG expressions in the heart tissue of lambs with WMD increased statistically compared to control animals similar to that reported by Yildirim et al., 2019 (1). We believe this increase in 8-OHdG immunoreactivity in lambs with WMD is likely a result of ROS-induced DNA damage. We think that ROS-induced lipid peroxidation and protein modifications as well as DNA damage play an important role in the pathogenesis of WMD.

In conclusion, this study revealed that ROS, which is an important factor in the pathogenesis of WMD, causes lipid peroxidation, protein modification and DNA damage. There is no literature data in which important oxidative stress markers such as 4-HNE, DT and 8-OHDG were evaluated together with IHC and IF methods in lambs with WMD. In this respect, we believe that the data obtained from this study will contribute to the pathogenesis of the disease and the literature data.

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