Determination of local expressions of IGF-1, LC3B and NF-kB in white muscle disease in lambs by immunohistochemical method

Research Article

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ABSTRACT

White muscle disease (WMD) is also known as Stiff Lamb Disease or Nutritional Muscular Dystrophy. Selenium and/or Vitamin E deficiency constitutes the etiology of the disease. This study aimed to immunohistochemically evaluate local protein expressions of Nuclear factor kappa B (NF-kB), Insulin-like growth factor-1 (IGF-1) and Microtubule-related protein 1A/1B-light chain 3 beta (LC3B) in WMD. The material of the study consisted of 15 WMD, and 6 healthy lamb heart samples. The heart tissues of the autopsied lambs were subjected to routine tissue processing and paraffin blocks were obtained. Then, it was stained with Hematoxylin-Eosin and immunohistochemical methods. Control group lambs had normal macroscopic appearance. Macroscopically, hyaline degeneration and zenker's necrosis, calcification areas were observed in WMD tissues. Microscopically, degenerative and necrotic muscle fibers, calcification areas, fibrosis, mononuclear cell infiltrates and macrophage infiltrates were detected in WMD heart tissues. Immunohistochemically, significant increases were detected in IGF-1 ($p<0.001$), LC3B ($p<0.001$) and NF-kB ($p<0.05$) in the WMD group compared to the control group. Immunoreactivity in the relevant primers was detected commonly in degenerative and necrotic muscle fibers. In addition, occasional immunoreactivity was observed in the relevant primers in inflammatory cell infiltrates. In conclusion, NF-kB, IGF-1 and LC3B protein expressions were evaluated immunohistochemically for the first time in lambs with WMD. Our findings show that IGF-1 and LC3B proteins are highly expressed in heart tissue in WMD. Additionally, it is possible to say that IGF-1 and LC3B can be used in the diagnosis of WMD.

Keywords: Histopathology, IGF-1, LC3B, NF-kB, white muscle disease

INTRODUCTION

White muscle disease (WMD) is also known as Stiff Lamb Disease or Nutritional Muscular Dystrophy. Selenium (Se) and/or Vitamin E (Vit E) deficiency constitutes the etiology of the disease. WMD is a major muscle degeneration disease of domestic animals (lambs, kids and calves) characterized by tissue destruction in striated muscles (Abutarbush and Radostits, 2003; Sobiech and Żarczyńska, 2020). WMD usually causes death due to heart failure in young animals. In WMD, degeneration, necrosis, fibrosis and calcification develop in the heart muscle. The incidence of WMD in our country is reported to be between 20-30%. The disease is generally seen in Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions (Karakurt et al., 2021; Karatas and Akcakavak, 2024; Yavuz, 2017).

Nuclear factor *kappa* B (NF-kB) is involved in various cellular processes such as cell proliferation and apoptosis, neurodevelopment, response to infection, inflammation (Zinatizadeh et al., 2021). In the

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resting state, NF-kB is inactivated in the cytoplasm by inhibitory proteins such as p105 and IkB in the cells. Reactive oxygen species (ROS) and some other free radicals affect these inhibitory proteins, causing the release of NF-kB and its migration to the nucleus (Biswas and Bagchi, 2016; Zinatizadeh et al., 2021).

Insulin-like growth factor-1 (IGF-1) is known as the essential mediator of growth hormone (GH). IGF-1 is part of a network of many growth factors, receptors and binding proteins involved in important processes such as cellular proliferation, differentiation and apoptosis (Bailes and Soloviev, 2021; Gusscott et al., 2016; Yoshida and Delafontaine, 2020). GH induces the production and release of IGF-1, which then binds to IGF-1R on the surface of cells. In addition, after the IGF-1-IGF-1R interaction, intracellular tyrosine kinase domains become activated. Thus, PI3K/Akt and Raf/MEK/ERK result in the activation of multiple signaling pathways (Gusscott et al., 2016; Yoshida and Delafontaine, 2020).

Autophagy is an intracellular catabolic process involving the degradation of intracellular components by lysosomes through the formation of autophagosomes. Autophagy plays important roles in maintaining cell homeostasis (Ichimiya et al., 2020). Microtubule-associated protein 1A/1B-light chain 3 (LC3), the mammalian homolog of yeast Atg8p, is considered an important component of autophagosomes (Meng et al., 2020). Although LC3 has several homologues in mammals, LC3B is the most commonly used for autophagy measurements. LC3B is expressed primarily in the heart, brain, skeletal muscle, and testis (Mizushima and Yoshimori, 2007; Wang et al., 2022).

In recent years, many studies have been carried out to elucidate the pathophysiology of WMD in domestic animals. For this purpose, different processes such as oxidative stress, apoptosis, etc. are evaluated (Karakurt et al., 2021; Tunca et al., 2009; Yildirim et al., 2019; Yumusak et al., 2018). Thus, new perspectives on the pathophysiology and diagnosis of WMD is revealed. This study aimed to immunohistochemically evaluate NF-kB, IGF-1 and LC3B protein expressions in WMD, a metabolic disease in lambs.

MATERIALS AND METHODS

Animal materials

The material of the study was heart tissue samples of 15 lambs (1-6 months, Merino) with WMD detected at necropsy and 6 healthy lambs (1-6 months, Merino) in Yozgat, Sivas and Konya regions.

Histopathological examination

Heart samples taken after necropsy were fixed in neutral formaldehyde for 24-48 hours. Afterwards, it was obtained in paraffin blocks by going through routine tissue tracking procedures. Sections were taken from paraffin blocks, stained with Hematoxylin-Eosin and examined under light microscopy (Luna, 1968).

Immunohistochemical examination

Sections were taken from paraffin blocks onto adhesive slides. Immunohistochemical examination was performed with the UltraVision Detection System Anti–Polyvalent, HRP (Ready–To–Use, TP–060–HL, Lab Vision, USA) kit in accordance with the manufacturer recommendations. Anti-LC3B (Santacruz Biotechnology, sc-271625, 1/200 dilution), Anti-IGF-1 (Santacruz Biotechnology, sc-518040, 1/200 dilution), and Anti-NF-kB (Bioss, Bs-0465R, 1/200 dilution) antibodies were used as primers. 3.3 diaminobenzidine (DAB) was used as chromogen and counterstaining was performed with Mayers-Hematoxylin. In the negative control, PBS was inoculated instead of antibody. Immunohistochemical scoring was performed semi-quantitatively (0; none, 1; mild, 2; moderate, 3; severe) (Akcakavak et al., 2023).

Statistical analysis

Evaluation of data between groups was done with SPSS (version 25.0, Inc., Chicago, USA) statistical program. Prior to analysis, immunohistochemical data were assessed for normal distribution and then subjected to independent sample t-test. The accepted significance limit was $p<0.05$.

RESULTS

Macroscopic results

Heart tissues of control group lambs had normal macroscopic appearance. Hyaline degeneration and zenker's necrosis, dystrophic calcification areas were observed in WMD heart tissues (Figure 1A). The relevant lesions were located in the endocardium and epicardium. Areas of hyaline degeneration and zenker's necrosis were generally pale in color and resembled fish and/or chicken flesh (Figure 1A-B). Dystrophic calcification areas were detected in the heart in 9 lambs.

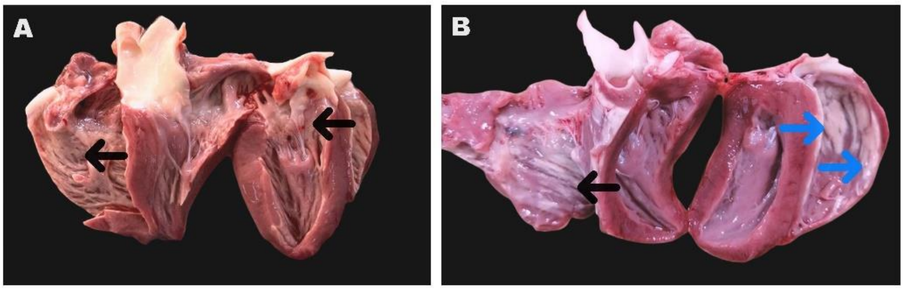

Figure 1. Macroscopic view of White muscle disease (WMD) heart tissues. **A.** Dystrophic calcification foci (arrows) in the endocardial section. **B.** Hyaline degeneration and zenker's necrosis (blue arrows), dystrophic calcification (black arrow) in the endocardium.

Microscopic results

Histopathological results

The heart of control animals showed normal structure (Figure 2A). Degenerative and necrotic muscle fibers were detected in WMD heart tissues. Striation was lost in these muscles, and they appeared swollen and pink (Figure 2B-C). Additionally, dystrophic calcification foci and areas of fibrosis were found occasionally. Mononuclear cell infiltrates were detected in the interstitial area and macrophage infiltrates were detected around necrotic muscle fibers (Figure 2D).

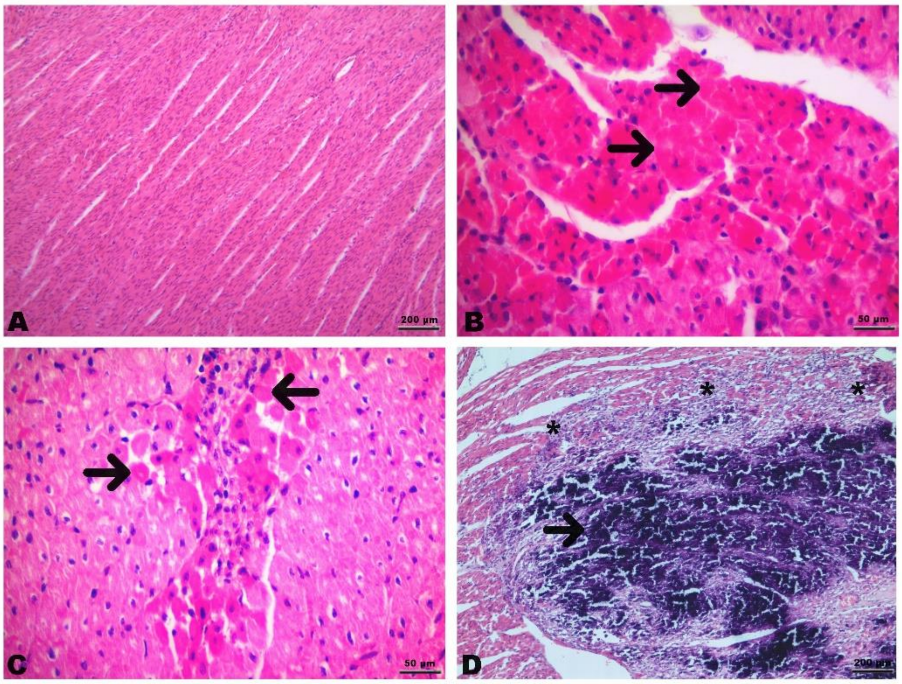

Figure 2. Histopathological examination of control and White muscle disease (WMD) lambs, Hematoxylin-Eosin, **A**. Normal histological appearance in control animals. **B-C.** Hyaline degeneration and zenker's necrosis (arrows) in lambs with WMD. **D.** Dystrophic calcification (arrow), fibrosis and inflammatory cell infiltration (stars) in WMD lambs.

Immunohistochemical results

Immunohistochemical scores for NF-kB, IGF-1 and LC3B are given in Table 1. In the control group, the immunoreactivity of the relevant primers was very mild or absent (Figure 3). Significant increases were detected in IGF-1 ($p<0.001$), LC3B ($p<0.001$) and NF-kB ($p<0.05$) in the WMD group compared to the control group. Immunoreactivity in the relevant primers was detected commonly in degenerative and necrotic muscle fibers. In addition, occasional immunoreactivity was observed in the relevant primers in inflammatory cell infiltrates (Figure 3).

Table 1. Statistical scores of IGF-1, LC3B and NF-kB in control and WMD lambs.

Primers	Control (n=6)	WMD (n=15)
IGF-1	0.83 ± 0.17^{b}	2.50 ± 0.22^{a}
LC3B	0.50 ± 0.22^{b}	2.17 ± 0.17^{a}
NF-kB	0.33 ± 0.21^{b}	1.5 ± 0.22^{a}

$^{a\text{-}b}$: Different letters on the line indicate statistical significance ($p<0.05$). WMD: White muscle disease, IGF-1: Insulin-like growth factor-1, LC3B: Microtubule-associated protein 1A/1B-light chain 3 beta, NF-kB: *Nuclear* factor *kappa* B.

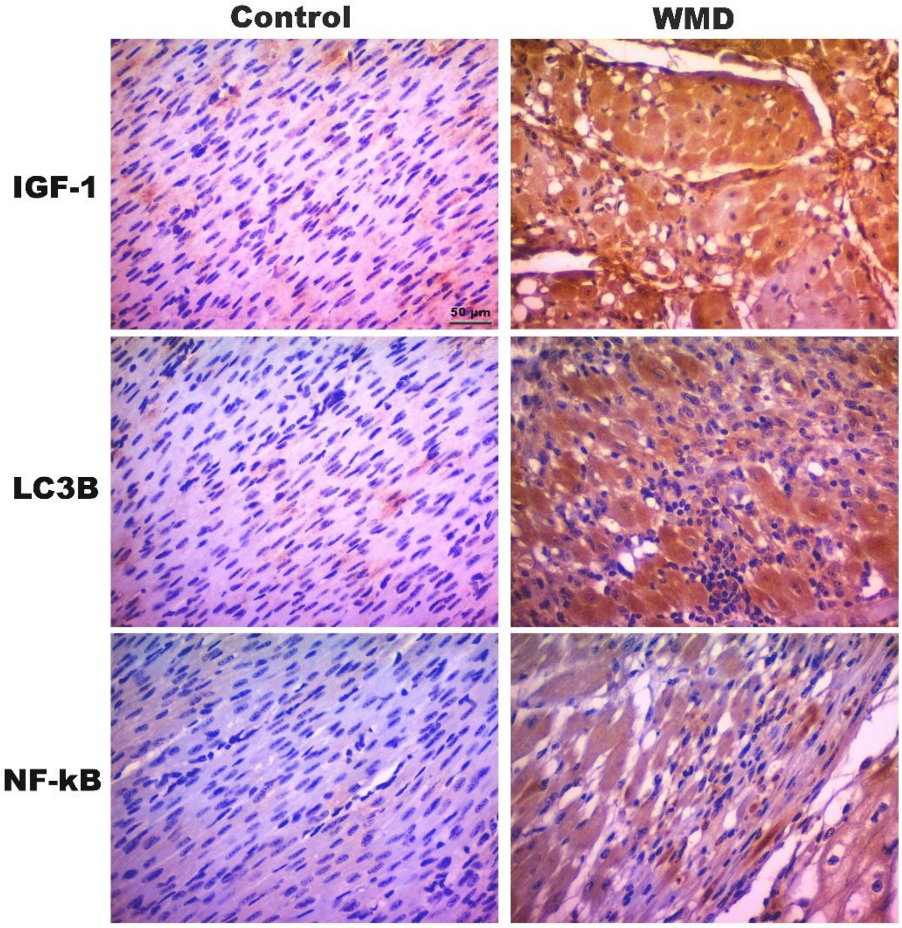

Figure 3. Immunohistochemical staining (DAB) of IGF-1, LC3B and NF-kB in WMD and control, bar; 50 µm. WMD: White muscle disease, IGF-1: Insulin-like growth factor-1, LC3B: Microtubule-associated protein 1A/1B-light chain 3 beta, NF-kB: *Nuclear* factor *kappa* B.

DISCUSSION

White muscle disease, which is an important metabolic disease of lambs, is frequently encountered in our country, especially in the Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions, and causes important economic loses in lamb breeding. Recently, many studies have been conducted on the pathophysiology, diagnosis and prognosis of WMD (Karakurt et al., 2021; Karatas and Akcakavak, 2024; Kozat et al., 2011; Kozat et al., 2007; Yumusak et al., 2018, Yıldırım et al., 2021). In this study, NF-kB, IGF-1 and LC3B protein expressions were revealed immunohistochemically in WMD in lambs and their effects on the pathophysiology of the disease were evaluated.

White muscle disease occurs in two different clinical forms: acute and subacute. The acute form is known as the cardiac form and is manifested by degeneration of the heart muscle and sudden death in young animals. The subacute form presents with skeletal muscle degeneration (Dabak et al., 2002). In the study conducted by Yavuz (2017) on 39 lambs, macroscopically, areas of pallor and calcification were detected in the heart, and microscopically, hyaline degeneration, zenker's necrosis, inflammation and calcification areas were detected in the heart. Karakurt et al., (2021) reported in their study that they detected macroscopic necrotic areas on the epicardial and endocardial surfaces and ventricular walls in lambs. They also reported that they detected microscopic degenerative and necrotic muscle fibers, inflammatory cell infiltrates and fibrosis. They also identified areas of calcification in necrotic muscle fibers. The macroscopic and microscopic findings of the current study are consistent with previous studies.

Some free radicals formed as a result of the decrease in antioxidant defense as a result of deficiency of Se and vit E cause oxidative stress. In Se and vit E deficiencies, lipid peroxidation and hydrogen peroxide cannot be cleared from the muscles due to the decrease in glutathione peroxidase (GSH-Px) activity. Moreover, ROS levels serve as markers of oxidative stress, and lipid peroxidation and imbalance of the redox system are associated (Ataollahi et al., 2013; Karakurt et al., 2021; Kozat et al., 2011; Kozat et al., 2007, Yıldırım et al., 2019). It has been reported in many studies that oxidative stress plays a very important role in the pathogenesis of WMD (Karakurt et al., 2021; Kozat et al., 2007; Yumusak et al., 2018; Yıldırım et al., 2019). In this context, oxidative stress is considered an important cause of degenerative and necrotic changes in relevant tissues.

Insulin-like growth factor-1 is a growth factor known as an anabolic and pro-myogenic factor important for the development and regeneration of skeletal muscle (Al-Shanti and Stewart, 2012). IGF-1 plays important roles in maintaining homeostasis in skeletal muscle by activating molecular steps critical for muscle homeostasis (O'Neill et al., 2015). It has been reported that increases in IGF-1 expression protect dystrophic muscle from necrosis (Grounds et al., 2008). Monocytes/macrophages recruited to the lesioned area after muscle injuries represent the first source of IGF-1 (Tidball and Welc, 2015). Previous studies have highlighted the importance of IGF-1 in promoting muscle regeneration through stimulation of myoblast proliferation and differentiation (Pelosi et al., 2007; Tonkin et al., 2015). Ye et al., (2013) attributed IGF-1 overexpression to alleviating muscle damage and accelerating muscle regeneration in their study. Sukhanov et al., (2007) reported in their study that increasing circulating IGF-1 reduced systemic and vascular oxidative stress. In the current study, IGF-1 expressions in WMD heart tissues were significantly higher than in control animals ($p<0.001$). IGF-1 expression was especially intense in degenerative and necrotic muscle fibers and macrophage cells. This situation has been interpreted as resulting from the body's response to the degenerative and necrotic damage occurring in the heart tissue due to WMD. Additionally, its intense expression in degenerative and necrotic muscle fibers may be caused by the response to oxidative stress.

Autophagy plays a role in important biological processes such as stress responses, programmed cell death and the elimination of damaged organelles. It is also known as an important sensor of the redox signal (Nichenko et al., 2016). It has been reported that the autophagy process tends to reduce oxidative stress (Yun et al., 2020). LC3 is known as a marker that plays an important role in the autophagy process (a component of the autophagosome). Many studies conducted in recent years state that the autophagy process increases after muscle damage and is critical for functional recovery and muscle renewal (Nichenko et al., 2016; Paolini et al., 2018). In the current study, it was determined that LC3B protein expressions were significantly increased in lambs with WMD compared to the control group ($p<0.001$). Current findings show that there may be upregulation of the autophagy process due to the oxidative stress process that occurs in WMD in lambs.

Nuclear factor kappa B is the transcription factor that regulates important processes such as inflammation and immune response (Zinatizadeh et al., 2021). Many studies have reported an increase in NF-kB expression after muscle damage and it is stated that it causes a worse pathology (Koshimizu et al., 2013; Nascimento et al., 2019; Wang et al., 2023). Oxidative stress and NF-kB are closely related and oxidative stress is known as an important inducer of NF-kB (Cuevas et al., 2005; Schreck et al., 1992). In the current study, increases in NF-kB levels were detected in lambs with WMD compared to the control ($p<0.05$), and it was thought that this situation may be due to oxidative stress. Because ROS and some other free radicals affect IkB inhibitor proteins, causing the release of NF-kB and its migration to the nucleus (Zinatizadeh et al., 2021).

The current study has some limitations. The most important limitation is the lack of blood concentrations values of the relevant proteins. Determination of blood concentrations of relevant proteins in future studies may provide a more comprehensive perspective on the diagnosis of WMD.

CONCLUSION

As a result, NF-kB, IGF-1 and LC3B protein expressions were evaluated immunohistochemically for the first time in lambs with WMD. Our findings show that IGF-1 and LC3B proteins are highly expressed in heart tissue in WMD. Additionally, it is possible to say that IGF-1 and LC3B can be used in the diagnosis of WMD.

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Ethical statement or informed consent: The study was approved by the Sivas Cumhuriyet University Animal experiments local ethics committee (02.02.2024, Decision no: 09).

Author Contributions: The study was designed by GA and MT. GA, OK, OD collected the relevant samples. ATC and OD laboratory performed tissue tracking procedures. GA and OK performed the immunohistochemical analyses. All authors read and approved the final version.

Availability of data and materials: All data and materials of the study are available in contact with the corresponsible author.

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