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Research Article

Determination of 8-OHdG and 4-HNE Expressions in Sheep with Hepatic Lipidosis by Immunohistochemical Method

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ABSTRACT

Lipidosis is generally defined as the accumulation of triglycerides in limited droplets within the cytoplasm of parenchymal cells. Lipidosis occurs due to toxic, chemical, infectious, and metabolic causes. This study aimed to reveal the local 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) expressions immunohistochemically according to the severity of the disease in sheep with hepatic lipidosis. The study material consisted of a total of 30 male sheep livers, including 6 healthy and 24 with hepatic lipidosis. After the liver samples were fixed in 10% formaldehyde solution, they underwent routine tissue processing to obtain paraffin blocks. Sections taken from the paraffin blocks were then subjected to Hematoxylin-Eosin (H-E) and immunohistochemical staining. Microscopically, control group liver samples showed normal histology. In livers with hepatic lipidosis, sharp-edged vacuoles of various sizes were detected in hepatocytes, and cell nuclei were pushed to the periphery. Additionally, focal hemorrhage and congestion, inflammatory cell infiltration in the portal area, bile duct proliferation, and connective tissue cells were observed. Microscopically, hepatic lipidosis cases were divided into two groups as moderate and severe based on the distribution of vacuoles in the section. In the immunohistochemical examination, 8-OHdG and 4-HNE expressions significantly increased in hepatic lipidosis cases compared to the control group ($p<0.001$). More intense immunoreactivity was detected especially in cases where disease severity increased ($p<0.001$). These results indicate that 8-OHdG and 4-HNE proteins play an important role in the pathogenesis of hepatic lipidosis and may be effective in increasing the severity of the disease.

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INTRODUCTION

Lipidosis is generally defined as the accumulation of triglycerides in limited droplets within the cytoplasm of parenchymal cells. Lipidosis occurs due to toxic, chemical, infectious, and metabolic causes (Erer et al., 2009; Jubb et al., 2012). Additionally, hypoxia, feeding with diets deficient in choline, ketosis, and abnormal pancreatic secretions constitute other causes. The two main factors known in the pathogenesis of lipidosis are the transport of lipids to the affected cell in amounts greater than it can metabolize and issues related to the synthesis of proteins and lipoproteins necessary for lipid transport. Lipidosis can frequently be observed primarily in the liver, as well as in the kidney and heart (Boden, 1997; Anderson and Borlak, 2008; Erer et al., 2009; Jubb et al., 2012).

Macroscopically, hepatic lipidosis presents with liver enlargement, blunting of edges, and a bulging cut surface, varying according to the severity of fatty change. The liver appears pale and yellowish in color. Microscopically, sharp-edged vacuoles of various sizes are observed in hepatocytes, and the cell nucleus is pushed to the periphery (Johnson et al., 1999; Erer et al., 2009; Jubb et al., 2012; Yeh and Brunt, 2014). Small droplet fatty change is seen in acute metabolic diseases, while large droplet fatty change is observed in toxic and some viral diseases. Hepatic steatosis can be diffuse or local based on its distribution. Centrilobular, peripheral, intermediate, and panlobular fatty changes are forms of diffuse fatty change (Jubb et al., 2012; Yeh and Brunt, 2014).

Hepatic lipidosis is defined as one of the significant metabolic disorders in animals that develops as a result of the abnormal presence of glucogenic and lipogenic products in the liver. It has been better described in large ruminants (Goff and Horst, 1997; Johnson et al., 1999; Al-Habsi et al., 2007). In large ruminants, it is associated with decreased feed intake, lactation, environmental cold, fetal growth, and negative energy balance caused by disease. Pregnancy toxemia, vitamin E deficiency, cobalt deficiency, toxication, and negative energy balance constitute important findings of hepatic steatosis in sheep (Ulvund, 1990, Johnson et al., 1999; Menzies et al., 2004; Al-Habsi et al., 2007). Furthermore, cobalt deficiency in sheep has been described to cause fatty hepatic degeneration, known as ovine white liver disease. It has been reported that hepatic lipidosis in sheep not only causes liver failure but also results in death in advanced stages (Johnson et al., 1999; Ulvund, 1990).

This study aims to demonstrate the 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) protein expressions according to the severity of hepatic lipidosis in sheep with hepatic lipidosis using the local immunohistochemical method.

MATERIAL AND METHODS

Animal material

The material for the study consisted of 24 sheep liver (Merino, Male, 6-24 months) samples that were positive for hepatic lipidosis, collected from different farms in Sivas and Yozgat provinces. Additionally, 6 healthy sheep (Merino, Male, 12-18 months) liver tissues were obtained from a nearby slaughterhouse.

Histopathological and Immunohistochemical evaluation

Liver samples were fixed in 10% neutral formaldehyde solution for 24-48 hours. Subsequently, paraffin blocks were obtained through routine processing, passing through alcohol and xylol series. Sections were taken from paraffin blocks onto slides, stained with Hematoxylin-Eosin (H-E), and examined under light microscopy (Akcakavak et al., 2024). Histopathological evaluation was performed semi-quantitatively by a blinded pathologist based on the presence of vacuoles in hepatocytes. Accordingly, vacuoles less than 50% were evaluated as mild (1), between 51% and 75% as moderate (2), and more than 75% as severe (3) (Johnson et al., 2004).

Immunohistochemical staining was performed using a commercial kit according to previously reported studies (Akcakavak et al., 2023; Kazak et al., 2024). 8-OHdG (Santa Cruz Biotechnology, sc-393871, 1/200 dilution, 1 hour incubation) and 4-HNE (Abcam, ab46545, 1/200 dilution, 1 hour incubation) antibodies were used as primary antibodies. 3,3 diaminobenzidine (DAB) was used as a chromogen, and after counterstaining with Mayer's hematoxylin, it was examined under a light microscope. Immunohistochemical

scoring was performed semi-quantitatively (0; none, 1; mild staining, 2; moderate staining, 3; severe staining, 4; very severe staining) (Kazak et al., 2024).

Statistical Analysis

Immunohistochemical findings obtained in the study were evaluated using One-way ANOVA and Duncan's test (SPSS, Inc., Chicago, USA 25.0) as post-hoc tests. The significance level was set at $p<0.05$.

RESULTS

Macroscopic Findings

Control group livers were found to have normal appearance and contour. In livers with hepatic lipidosis, enlargement in size, fragility, blunting of edges, and paleness in color were detected. Cut surfaces had a swollen appearance, and paleness was prominent throughout the parenchyma. Petechial hemorrhages were detected on the liver serosa in 2 cases (Figure 1). Additionally, parasite cysts were observed in 3 different cases in the liver, and parasite migration paths were seen in the liver parenchyma in 2 different cases.

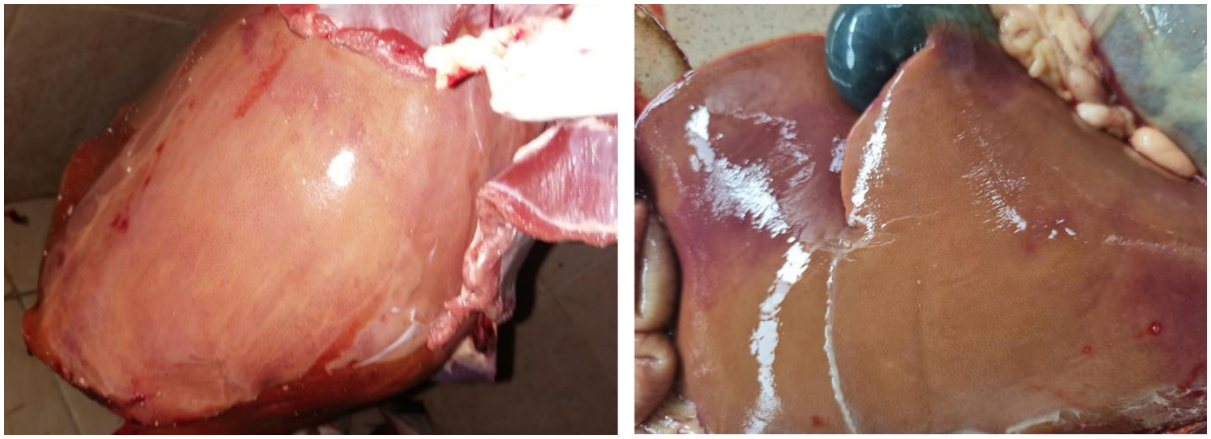

Figure 1. *Macroscopic appearance of pale livers from different sheep.*

Microscopic Findings

Control group liver tissues showed normal histology. In liver samples with hepatic lipidosis, sharp-edged, variously sized vacuoles were detected in hepatocytes, and cell nuclei were pushed to the periphery. Hepatocyte nuclei occasionally showed necrotic changes. Additionally, focal hemorrhage and congestion, inflammatory cell infiltration in the portal area, bile duct proliferation, and connective tissue cells were detected (Figure 2). Moreover, fibrosis foci were found in 6 severe (score 3) cases. In the histopathological evaluation, 7 cases were found to be moderate (score 2) and 17 cases were severe (score 3).

The immunohistochemical statistical scores between the groups are given in Table 1. Immunohistochemically, no 8-OHdG immunopositivity was observed in control group livers. However, 4-HNE immunoreactivity was very mild and/or absent. In livers with hepatic lipidosis, 8-OHdG and 4-HNE expressions were found to be significantly increased (Figure 3). Immunoreactivity in related primers (8-OHdG and 4-HNE) showed cytoplasmic localization. Especially in severe cases (score 3), 8-OHdG and 4-HNE immunoreactivity was more intense and widespread compared to moderate cases (score 2) ($p<0.001$).

Table 1. *Immunohistochemical statistical scores of healthy and hepatic lipidosis livers (Mean+SE).*

Primers	Control (n;6)	Moderate (score 2) (n;7)	Severe (score 3) (n;17)
8-OHdG	0.00 ± 0.00^{c}	1.28 ± 0.19^{b}	2.33 ± 0.28^{a}
4-HNE	0.33 ± 0.21^{c}	1.57 ± 0.20^{b}	2.55 ± 0.17^{a}

[a-c] Letters in the same row indicate statistical significance ($p<0.001$). (8-OHdG; 8-hydroxy-2'-deoxyguanosine, 4-HNE; 4-hydroxynonenal)

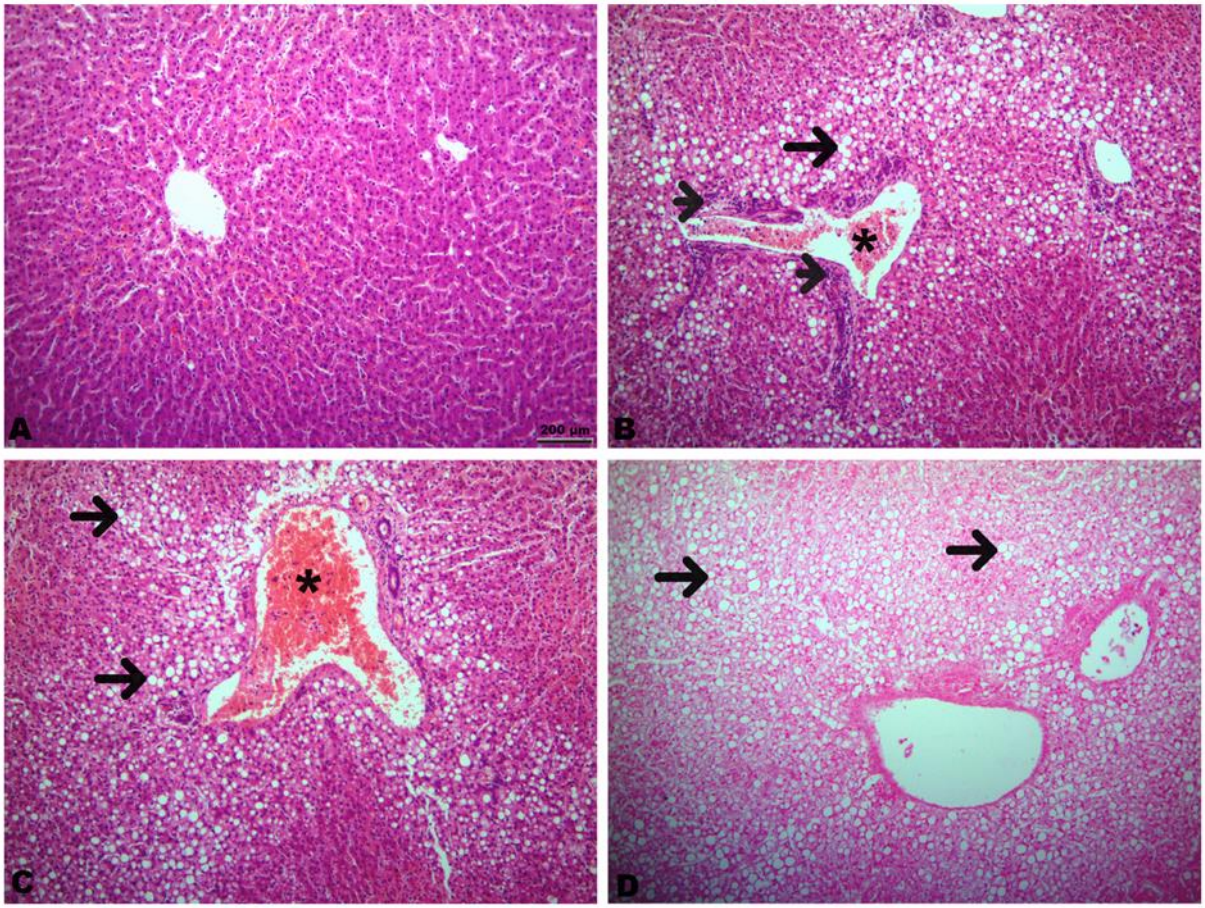

Figure 2. *Microscopic appearance of healthy and hepatic lipidosis livers, Hematoxylin-Eosin (H-E), x100.* ***A.*** *Normal histological appearance of control group liver.* ***B-C.*** *Appearance of liver samples with moderate diffuse (score 2) vacuoles.* ***D.*** *Appearance of liver samples with severe diffuse (score 3) vacuoles. (vacuole appearance (arrows), inflammatory cell infiltration (arrowheads), congestion (asteriks)*

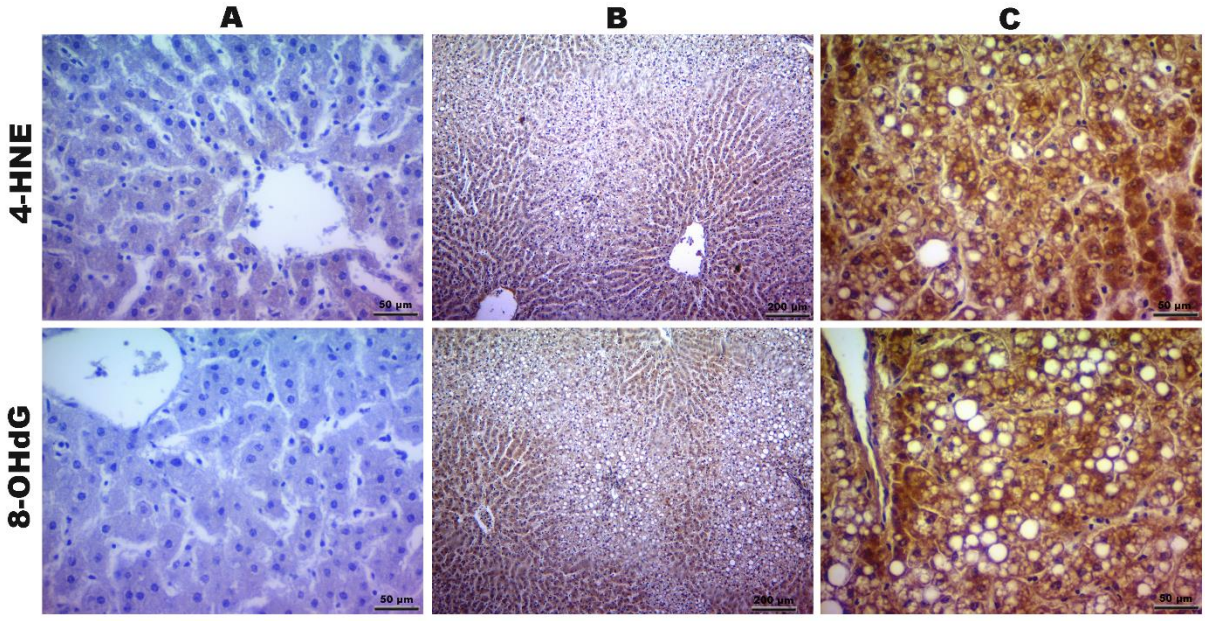

Figure 3. *Microscopic appearance of healthy and hepatic lipidosis livers, immunohistochemical staining (DAB). (A; Control group, B; Liver with moderate diffuse (score 2) vacuoles, C; Liver with severe diffuse (score 3) vacuoles, 4-HNE; 4-Hydroxynonenal, 8-OHdG; 8-hydroxy-2'-deoxyguanosine).*

DISCUSSION

The liver is known as the body's largest metabolic center. It has important functions such as storage and control of carbohydrate metabolism, synthesis of plasma proteins, detoxification of various drugs and toxins, urea production, and fat metabolism. As the liver is the main organ of fat metabolism, hepatic lipidosis condition is frequently encountered. It is also constantly exposed to toxic, chemical, and infectious effects (Thapa and Walia, 2007; Erer et al., 2009). Studies in cattle report that liver fatty cases are encountered at rates of 4-13% (Oruc, 2009; Altun and Saglam, 2014). However, it is stated that the incidence of liver fatty in sheep is lower and is associated with an extensive feeding style. Gözün and Kıran (1999), reported that they detected fatty cases at a rate of 0.38% in 1561 sheep livers with lesions. In addition to the limited number of studies on ovine hepatic lipidosis cases in the literature review, research is more focused on pregnancy toxemia (Iqbal et al., 2022; Ji et al., 2023). In the current study, 8-OHdG and 4-HNE protein expressions were determined immunohistochemically according to the severity and prevalence of steatosis in male sheep with hepatic lipidosis.

In cases of hepatic lipidosis in small ruminants, macroscopically, enlargement, brittle structure and pale color of the liver are reported. Histopathologically, sharp-edged fat vacuoles in hepatocytes, inflammatory cell infiltration in the portal area, bile duct proliferation, and fibrosis are reported (Ulvund, 1990, El-Khodery et al., 1994; Johnson et al., 1999; Al-Habsi et al., 2007). The macroscopic and microscopic findings observed in the present study, especially in severe cases (score 3), exhibited similar findings to the literature. In addition, the detection of occasional fibrosis foci in 6 severe (score 3) cases indicates an advanced state of the disease. Moreover, macroscopic findings related to parasite infestation were found in 5 cases.

8-OHdG is defined as a marker of oxidative DNA damage caused by reactive oxygen species. 8-OHdG is produced from guanine in DNA bases through oxidative stress. 8-OHdG is an important marker that plays a role in the pathogenesis of inflammatory, diabetic, and autoimmune diseases and malignancies (Miyamoto et al., 2011; Varghese et al., 2020). Miyamoto et al. (2011) reported in their study investigating the relationship between metabolic risk factors and oxidative stress in humans that hypertriglyceridemia may be associated with hyperoxidative stress evaluated by 8-OHdG. It is also stated that hypertriglyceridemia can stimulate ROS production and impair the antioxidant defense system (Hiramatsu et al., 1991; Araujo et al., 1995). Additionally, different human studies report that oxidative stress markers such as 8-OHdG and 4-HNE are found in high concentrations in non-alcoholic fatty liver disease (NAFLD). Furthermore, NAFLD is characterized by increased microsomal fatty acid oxidation, which is associated with greater ROS production (Seki et al., 2002; Sumida et al., 2021). Dinçel et al. (2018) reported in their study on streptozotocin-induced type-1 diabetic rats that 8-OHdG expressions significantly increased in the diabetic group compared to the control group, and oxidative DNA damage plays important roles in diabetes-related liver degenerations. In the present study, 8-OHdG expressions were evaluated in hepatic lipidosis in sheep, and it was found that 8-OHdG immunoreactivity increased as the severity of the disease in the liver increased. Previous studies have reported that oxidative stress is upregulated in hepatic lipidosis cases (Yoshino et al., 1992 ; Webb and Twedt, 2008; Elshafey et al., 2023). This suggests that oxidative stress-induced oxidative DNA damage may play an important role in the disease process in hepatic lipidosis in sheep.

In conditions of increased metabolic disorder and inflammation, ROS production may exceed the cell's antioxidant capacity. The resulting free radicals can react with various amino acids and lipids (Herndon et al., 2014). Another oxidative reaction caused by excessive ROS levels is lipid peroxidation. 4-HNE is known as the cytotoxic end product of lipid peroxidation (Yang et al., 2003; Ayala et al., 2014). Lipid peroxidation is thought to play a role in the progression of many diseases, particularly those characterized by lipid accumulation such as NAFLD. In a study conducted by Fu et al. (2022), it was reported that an n-6 PUFA diet had a negative effect on alcohol-induced liver damage and steatosis, and they suggested that this might be related to the upregulation of 4-HNE. In the present study, the highest 4-HNE immunoreactivity was found in severe (group 3) cases, and this indicates that lipid peroxidation may play important roles in the progression of the disease in the pathogenesis of hepatic lipidosis in sheep.

CONCLUSION

In conclusion, in the current study, local expressions of 8-OHdG and 4-HNE were evaluated immunohistochemically in sheep with hepatic lipidosis. The present results show that 8-OHdG and 4-HNE

proteins play an important role in the pathogenesis of the disease and may play an active role in increasing the severity of the disease.

AUTHOR CONTRIBUTION

All authors contributed equally.

ETHICAL STATEMENT

Scientific rules, ethics and citation rules were followed during the writing process of the study titled "**Determination of 8-OHdG and 4-HNE expressions in sheep with hepatic lipidosis by immunohistochemical method**"; There was no tampering with the data collected and this study was not sent to any other academic publication environment for evaluation. The necessary ethics committee permissions were obtained by Sivas Cumhuriyet University Animal Experiments Ethics Committee's Decision dated 16.08.2024 and numbered 2024/55.

CONFLICT OF INTEREST

The authors certify that they have no conflict of interest.

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