Immunohistochemical evaluation of CD68, β-Catenin, α-SMA and Ki67 expression in Kupffer and parenchymal stellate cells associated with bovine liver lesions leading to fibrosis

Abstract

Liver fibrosis is a disorder resulting from numerous diseases that threaten animal life. Over two years (2021-2023), a total of 1,525 bovine livers were inspected, and common liver diseases leading to fibrosis, including fascioliasis, fatty change, hydatid cyst, and abscess, were diagnosed using various histochemical staining techniques. The evaluation of serum enzymes indicated a significant increase in ALT in fascioliasis, as well as AST and GGT in fascioliasis and fatty change, compared to other groups (P<0.05). Immunohistochemical results demonstrated that the expression intensity and mean number of α-SMA-positive stellate cells and β-catenin significantly increased (P<0.05) in fascioliasis, fatty change, abscess, and hydatid cyst lesions compared to normal liver. The expression pattern of α-SMA in lesions was observed in three states: perisinusoidal, periportal, and pericentral. Furthermore, in fatty liver change, nuclear expression of β-catenin was observed in parenchymal cells. Indeed, unlike the human liver, where β-catenin expression is present in bile duct cells under normal conditions, in cattle, only membranous-cytoplasmic expression of β-catenin was recorded in bile duct cells of livers affected by fascioliasis. The number of CD68-positive Kupffer cells and Ki67-positive cells in liver lesions showed a significant increase compared to normal liver (P<0.05). Overall, considering the results, with increasing severity of liver fibrosis, the expression of CD68, β-catenin, α-SMA, and Ki67 markers also increases. In other words, with the onset and progression of inflammation in the bovine liver, simultaneous activation of stellate and Kupffer cells and increased collagen production contribute to the reconstruction of the damaged liver with connective tissue, thereby leading to liver fibrosis.

Keywords: Bovine liver, CD68, β-Catenin, α-SMA, Fibrosis

1. Introduction

The process of liver fibrosis involves the interaction of various liver cells, including hepatocytes, stellate cells, parenchymal cells, and cholangiocytes. It also involves the release or secretion of various growth factors, such as TGF-β. These growth factors activate signaling in hepatic stellate cells (HSCs) and are paracrine secreted by Kupffer cells in a network-like chain (1). Cirrhosis is a form of liver fibrosis that develops due to chronic hepatitis and other liver diseases, such as viral hepatitis, alcoholic liver damage, non-alcoholic steatohepatitis, and autoimmune liver diseases. It is linked to numerous complications and has a bleak prognosis. Therefore, it will be important to combat cirrhosis (fibrosis) by developing better therapies. The Wnt/β-catenin pathway is a critical regulator of cell growth and proliferation and is important in normal liver development (2). Activation of the Wnt/β-catenin pathway prevents the regeneration of hepatocytes by replacing the extracellular matrix (ECM), leading to the appearance of scar tissue and the formation of regenerated nodular hepatocytes, resulting in the loss of the primary function and health of hepatocytes. Selective inhibition of β-catenin prevents inflammatory processes since chemokines and pro-inflammatory cytokines are produced during Wnt activation, resulting in a decrease in activated stellate cell growth, reduced collagen production, and decreased progression of liver fibrosis within the body (3). It has been proven that Wnt/β-catenin signaling is involved in the fibrosis of several organs, such as the kidney, lung, skin, and liver. The role of macrophages in liver fibrosis has also been confirmed. Macrophages, having a dual role, can simultaneously affect both the injury caused by fibrosis and the promotion of repair in a damaged organ. Furthermore, it has been reported that the specific deletion of β-catenin by macrophages, which is due to a defect in the migration and fibroblast adhesion and the production of TGF-β, has led to incomplete healing of skin wounds (4). The progression of fibrogenic chronic liver disease, regardless of its cause, is characterized

by chronic parenchymal damage, chronic activation of the inflammatory response, and persistent activation of liver fibrogenesis and the pathological wound healing response. It has been established that upon liver injury, HSCs undergo an activation process that enables them to acquire a myofibroblast phenotype. The biological activities of myofibroblasts (MFs) are vital for liver tissue repair and fibrogenesis. Following this signaling stage, fibrogenic TGF-β in quiescent hepatic stellate cells becomes activated. The initial source of TGF-β is paracrine, including hepatocytes, Kupffer cells, and activated platelets in response to liver injury (1). In liver fibrogenesis, an important role is played by hepatic MFs, a heterogeneous population of smooth muscle actin-positive cells that originate from various precursor cells through a process of activation and transdifferentiating (5). Kupffer cells (KCs) are specialized macrophages residing in the liver and belonging to the mononuclear phagocytic system. In acute liver injuries, KCs release inflammatory cytokines, such as inducible nitric oxide synthase (iNOS), through direct contact between hepatocytes and cells. Subsequently, they effectively eliminate pathogens by releasing nitric oxide (NO). Conversely, KCs secrete substantial amounts of the profibrogenic cytokine transforming growth factor-beta 1 (TGF-β1), which enhances the activity and proliferation of (HSCs), marking the activation of liver fibrosis and ultimately leading to the development and progression of hepatic fibrosis. HSCs, in turn, further increase the proliferation and differentiation of KCs through paracrine effects (6). In a study evaluating hepatic lipidosis in slaughtered cattle in Urmia, Iran, it was reported that the most prevalent pathological liver lesions were, in descending order, fascioliasis, fatty change, and hydatidosis. Elevated activities of GGT, ALT, and AST in the liver are often indicative of suspected acute and chronic liver diseases (7). Determination of AST and GGT activities in dairy cattle is commonly associated with fatty liver syndrome. Increased serum AST activity is a sensitive indicator of liver injury, even if the damage is subclinical in nature. Unlike in pigs, hepatocytes in ruminants do not exhibit high ALT activity; however, an elevation in serum ALT activity

during liver injury and even necrosis is significant. GGT is a membrane-bound enzyme involved in secretory and absorptive functions in the organs, and its plasma levels are noteworthy for hepatobiliary diseases. This enzyme is associated with cholestasis and is utilized in the diagnosis of liver disease. Its activity is relatively high in the livers of cattle, horses, and sheep (8). In contrast to AST, serum GGT concentration is a specific indicator of liver injuries, rendering it a useful diagnostic criterion (9). The present study aimed to investigate the relationship between stellate cells, Kupffer cells, hepatocytes, and cholangiocarcinoma cells in lesions leading to liver fibrosis through biochemical and pathological examinations of common liver lesions in slaughtered cattle at the Urmia abattoir. Immunohistochemical markers SMA-α and CD68 were employed to differentiate stellate cells and Kupffer cells (macrophages), respectively. Additionally, the expression pattern and role of the β-catenin marker in healthy and damaged livers were studied and compared with the aforementioned markers to evaluate the expression levels of these markers in common bovine liver lesions, such as fascioliasis, hydatidosis, and fatty change, which can lead to fibrosis. Furthermore, the Ki67 antibody (a nuclear proliferation marker) was employed to evaluate cellular proliferation in the aforementioned lesions.

2. Materials and Methods

2.1. Sample Collection

The present study was conducted over a two-year period from 23 July 2021 to 23 July 2023, during which livers from 1,525 slaughtered cattle at the Urmia industrial slaughterhouse were inspected and sampled. The livers were carefully examined macroscopically (visually) for their appearance, tissue consistency, color, shape, and condition of the liver margins. It is noteworthy that all livers were inspected for the presence of hydatid cysts and abscesses (1). Regarding livers with hydatid cysts or abscesses, only those exhibiting more severe lesions, higher

numbers, and larger areas of liver involvement accompanied by hardening of the liver tissue were subjected to pathological and immunohistochemical evaluations. Since different grades of fatty change and fascioliasis can be assessed microscopically, 50 liver tissue samples were selected and microscopically evaluated based on visual assessments. For each lesion type, whose grade and severity were determined microscopically and by H&E staining, 10 samples of severe fatty liver, 10 samples of grade III fascioliasis, 10 samples of hydatid cysts, and 10 samples of abscesses were selected for immunohistochemical evaluations. Grading of fatty change in affected livers was performed based on the presence of lipid vacuoles in hepatocytes as follows: (a) normal liver with less than 5% of hepatocytes containing lipids, (b) mild (5-33%), (c) moderate (33-66%), and (d) severe (>66%) (7). Additionally, grading of liver lesions due to cholangiohepatitis resulting from fascioliasis was conducted as follows: (a) grade I: simple inflammatory reaction with periportal infiltration into the hepatic parenchyma, (b) grade II: distinct inflammatory reaction, telangiectasia, central vein congestion, degenerative or necrotic changes in hepatocytes, and the presence of the parasite in the biliary system, and (c) grade III: chronic inflammatory reaction with centrilobular necrotic or degenerative changes, pericellular fibrosis, portal areas with metaplasia, hyperplasia, and dilation of bile ducts containing Fasciola parasites (10). Masson's trichrome and PAS stains were employed for the differentiation of connective tissue and lipids, respectively, in various liver lesions.

2.2. Evaluation of AST, ALT, and GGT Enzymes

Blood was collected from the jugular vein of the test cattle into heparinized tubes to measure the levels of AST, ALT, and GGT enzymes and used as an anticoagulant to measure the levels of AST, ALT, and GGT enzymes. After separating the plasma by centrifugation at 1,500 rpm for 15 minutes at 20°C, the enzyme levels were measured using a spectrophotometer (8).

2.3. Immunohistochemistry

The avidin-biotin complex (ABC) peroxidase method was employed for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues. Briefly, 4 µm thick tissue sections were deparaffinized and rehydrated through descending grades of ethanol. Antigen retrieval was performed by boiling in sodium citrate buffer up to 100°C. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide for 15 minutes, and non-specific binding was blocked with diluted horse serum (1:100) for 1 hour. The primary antibodies used were α-SMA (Dako, USA; 1:200), CD68 (Dako, Denmark, Clone KP1; 1:150); Ki67 (Dako, Denmark, 1:100) and β-catenin (Dako, Denmark, Clone β-Catenin-1, 1:100). Biotinylated goat anti-rabbit IgG antibody and horse anti-mouse IgG antibody were used as secondary antibodies. After incubation with primary and secondary antibodies, the slides were stained with diaminobenzidine and counterstained with hematoxylin. Negative controls were prepared by omitting the primary antibody, and appropriate positive control tissues were included for each run (11). The intensity and percentage of parenchymal cells showing positive reactions for β-catenin, αSMA, CD68, and Ki67 were evaluated in random microscopic fields for immunohistochemical scoring. At least 300 hepatic cells were counted in each field at 40× magnification for each sample, and the average number of positive cells was calculated for comparison among common liver lesions (11). The percentage of positive cells was scored as follows: (0) no staining, (1) positive staining in ≤25% of the cells, (2) positive staining in 26-50% of cells, (3) positive staining in 51-75% of cells, and (4) positive staining in >75% of the cells (12).

2.4. Statistical Analysis

Statistical analysis of the obtained data was performed using SPSS software version 27. One-way analysis of variance (ANOVA) was used to analyze the serum levels of AST, ALT, and GGT enzymes across different lesions, and the two-sided Dunnett's t-test was employed for pairwise comparisons among groups. The normality of the count data for the number of positive

cells for αSMA, CD68, β-catenin, and Ki67 in hepatic parenchymal cells affected by different liver lesions was assessed using the Kolmogorov-Smirnov test. If the residuals were normally distributed, one-way ANOVA and Tukey's post hoc test were used for comparisons between groups. Data are presented as Mean ± SD, and $P<0.05$ was considered statistically significant.

3. Results

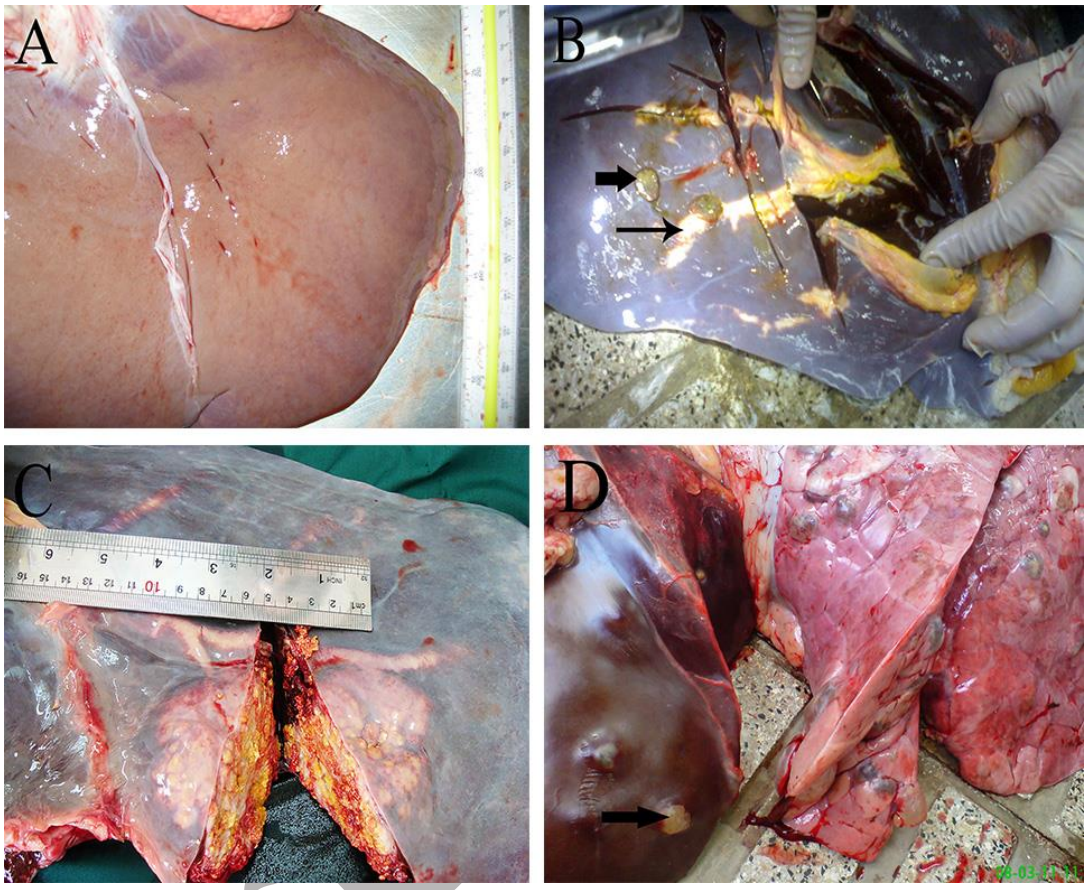
3.1. Gross and Microscopic Pathology

During the two years from 23 July 2021 to 23 July 2023, livers from 1,525 slaughtered cattle were inspected. Of these, 822 (53.90%) were bulls, and 703 (46.09%) were cows. Their age distribution was as follows: ≤1.5 years, 2.5 years, 3.5 years, 4.5 years, and older. Based on the slaughterhouse examination results, 89 (9.89%) livers from 822 bulls and 77 (10.95%) livers from 703 cows had hydatid cysts. Additionally, 16 (1.94%) livers from 822 bulls and 20 (2.84%) livers from 703 cows had the abscesses. Fifty tissue samples were selected from the 1,525 inspected livers that appeared normal macroscopically or exhibited hydatid cysts, fascioliasis, abscesses, or fatty change. Histopathological sections were prepared from these samples and stained with hematoxylin and eosin (H&E). The grading results for cholangiohepatitis due to fascioliasis and fatty change in the 50 microscopically examined liver samples are presented in Table 1.

Table 1. Abundance of hepatic fatty change and cholangiohepatitis (fascioliasis) in different age groups detected by microscopic grading of bovine livers

Lesion (n=50)		Age (year)			
		≤1.5	2.5	3.5	≥4.5
Fatty change	Normal	1 (%50)	5 (%50)	3 (%15.8)	3 (%15.8)
	Mild (%5-%33)	1 (%50)	3 (%30)	6 (%31.6)	5 (%26.3)
	Moderate (%33-%66)	0 (%0)	2 (%20)	5 (%26.3)	4 (%21.1)
	Severe (>%66)	0 (%0)	0 (%0)	5 (%26.3)	7 (%36.8)
Cholangiohepatitis by Fasciola	Grade I	0 (%0)	1 (%100)	6 (%32.7)	10 (%37)
	Grade II	0 (%0)	0 (%0)	12 (%54.5)	10 (%37)
	Grade III	0 (%0)	0 (%0)	4 (%18.2)	7 (%26)

١٦٧ During the gross examination of slaughtered cattle livers and necropsy, common liver lesions
١٦٨ such as fatty change, fascioliasis, abscesses, and hydatid cysts were observed, as shown in
١٦٩ Figure 1.



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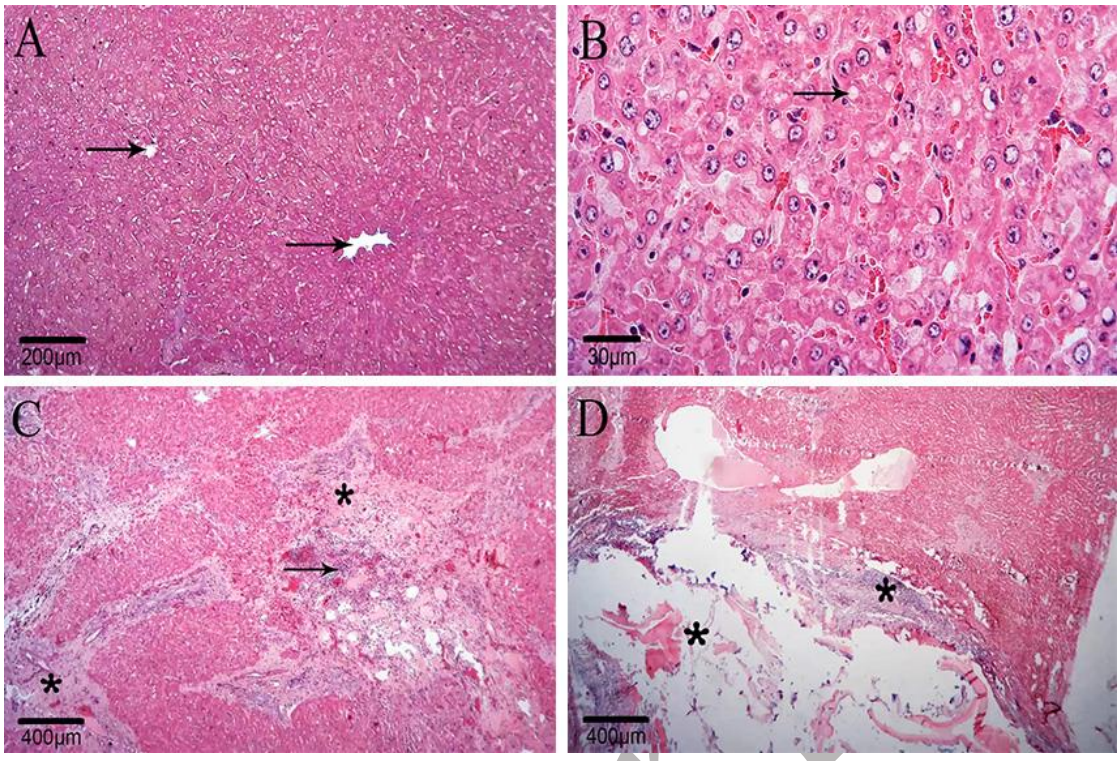
١٧١ Figure 1: Some of common liver lesions observed at the slaughterhouse. (A) Fatty change with
١٧٢ pale yellowish discoloration in a liver. (B) The liver is affected by fascioliasis, with the
١٧٣ migration pathways of the parasites visible as thin white lines (thin arrow). Additionally, several
١٧٤ Fasciola specimens (thick arrow) that have emerged from the bile ducts are observed. (C) A
١٧٥ large hepatic abscess containing pus, connective tissue, and calcification with a relatively firm
١٧٦ consistency. (D) Hydatid cysts (arrow) of varying sizes contain clear fluid in the lungs and liver.

١٧٧ The initial diagnosis of common bovine liver lesions, including fatty change, fascioliasis,
١٧٨ abscesses, and hydatid cysts, was performed using hematoxylin and eosin (H&E) staining, as
١٧٩ presented in Figure 2.



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١٨١ Figure 2: Photomicrographs of common bovine liver lesions leading to fibrosis. (A) Normal
١٨٢ liver with a regular hepatocyte structure and central veins (arrow). (B) Hepatic fatty change,
١٨٣ with clear vacuoles within hepatocytes (arrow). (C) Affected liver by fascioliasis, with fibrotic
١٨٤ areas (asterisk) inflammatory cell aggregation (predominantly lymphocytes) (arrow). (D) A
١٨٥ hepatic abscess containing a collection of inflammatory neutrophils (asterisk). (H&E).

١٨٦ To confirm and differentiate lipid and glycogen, periodic acid-Schiff (PAS) staining was
١٨٧ employed. Additionally, Masson's trichrome staining was used to evaluate and confirm fibrosis
١٨٨ and collagen fiber density, with collagen and connective tissue appearing blue in the slides
١٨٩ (Figure 3).

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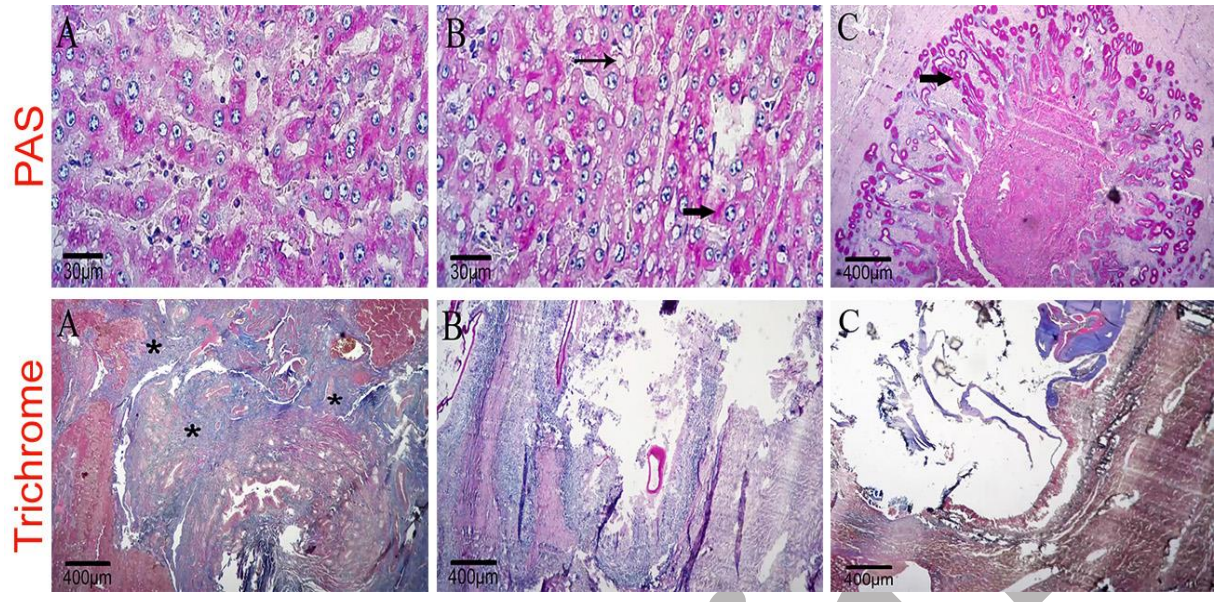

Figure 3: Microscopic sections of bovine liver for the differentiation of fat, glycogen and connective tissue. (A) The normal liver has a regular structure with small amounts of glycogen accumulation in some cells as pale pink areas without fat vacuoles. (B) Liver with fatty change displays numerous clear, unstained fat vacuoles (thin arrow) and some glycogen accumulation (thick arrow) in certain cells. (C). The liver is affected by fascioliasis with bile duct hyperplasia an increase in mucous glands containing PAS-positive cells (thick arrow). PAS. (A) The liver affected by fascioliasis with severe connective tissue (fibrosis) stained blue (asterisk) in most tissue areas. (B) A hepatic abscess containing inflammatory cell infiltration and an increase in connective tissue. (C) Liver with a hydatid cyst, displaying inflammatory cell infiltration and a small amount of connective tissue around the cyst. (Masson's trichrome).

3.2. Blood Biochemistry

The results of the one-way analysis of variance test for the levels of AST, ALT, and GGT enzymes in different liver groups (normal, fascioliasis, fatty change, hydatid cyst, and abscess), as well as the two-sided Dunnett t-test for comparing them between different groups in terms of Mean ± SD are presented in Table 2. According to this table, a significant increase (P<0.05) in ALT enzyme was observed in the fascioliasis group, and a significant increase (P<0.05) in

AST and GGT enzymes was observed in the fascioliasis and fatty change groups compared to the normal and other liver lesion groups.

Table 2. Measured levels of blood ALT, AST and GGT in bovine with different liver lesions. Data are presented as Mean ± SE and $P<0.05$ is significant

Enzyme	Group (n=10)	Mean ± SE	Lower bound	Upper bound	Minimum	Maximum	P value
ALT	Normal	25.83 ± 1.89	21.54	30.11	18.70	34.20	-
	Fascioliasis	45.45 ± 1.71*	41.56	49.33	38.10	53.20	0.000
	Fatty change	29.27 ± 2.10	24.51	34.02	21.80	39.90	0.518
	Hydatid cyst	24.89 ± 1.67	21.11	28.66	17.40	32.60	0.990
	Abscess	28.32 ± 2.07	23.62	33.01	19.80	37.70	0.762
AST	Normal	70.45 ± 2.88	63.92	76.97	57.10	81.50	-
	Fascioliasis	139.76 ± 9.35*	118.59	160.92	99.50	179.40	0.000
	Fatty change	111.70 ± 6.26*	97.52	125.87	88.40	144.60	0.000
	Hydatid cyst	75.13 ± 2.91	68.53	81.72	64.10	90.20	0.937
	Abscess	74.84 ± 3.71	66.43	83.24	59.20	91.50	0.949
GGT	Normal	16.99 ± 0.55	15.73	18.24	14.50	19.30	-
	Fascioliasis	26.50 ± 1.22*	23.73	29.26	19.70	32.40	0.000
	Fatty change	23.20 ± 0.89*	21.18	25.21	19.60	27.30	0.000
	Hydatid cyst	16.83 ± 0.82	14.96	18.69	12.80	20.90	1.000
	Abscess	17.03 ± 0.80	15.20	18.85	13.70	22.70	1.000

Note: Asterisk (*) indicates a significant increase of the enzyme related to a lesion group compared with the normal one.

3.3. Immunohistochemistry

Table 3 shows the mean values ± the standard error of the number of cells positive for CD68, catenin, α-SMA, and Ki67 in liver cells affected by different lesions. The mean number of cells with positive immunoreaction to α-SMA (stellate cells) significantly increased ($P<0.05$) in all groups with liver lesions compared to the normal group, with the fascioliasis group showing the highest increase among the other groups. In addition, a significant increase ($P<0.05$) in the mean number of cells positive for catenin immunoreactivity was observed in the fascioliasis and hydatid cyst groups compared to the normal group. In addition, the mean number of cells positive for CD68 (Kupffer cells) and Ki67 (indicating cell proliferation) showed a significant increase ($P<0.05$) in the fascioliasis and hydatid cyst groups compared to the normal group.

Table 3: Comparison of Mean ± SE of the number of cells with positive reaction against αSMA, β-Catenin, CD68 and Ki67 in liver parenchymal cells affected by different liver lesions

Group	Antibody			
	αSMA	βcatenin	CD68	Ki67
Normal liver	35.41±2.27[a]	7.60±0.65[a]	0.18±0.04[a]	0.33±0.07[a]
Fascioliasis	137.52±3.20[e]	185.85±3.31[c]	3.67±0.58[b]	2.44±0.38[b]
Fatty change	89.91±3.33[d]	13.31±1.27[a]	2.85±0.38[b]	2.25±0.21[b]
Hydatid cyst	59.07±3.09[c]	69.14±4.50[b]	0.46±0.09[a]	0.16±0.04[a]
Abscess	47.80±2.76[b]	16.71±1.24[a]	0.27±0.08[a]	0.08±0.02[a]
P value	<0.001	<0.001	<0.001	<0.001

Note: Different letters in each column indicate the existence of a significant difference at the level of $P<0.05$ between the tested treatments in Tukey test.

The results of immunohistochemical labelling for α-SMA, β-catenin, CD68 and Ki67 are presented in Figures 4 and 5. Accordingly, the results for α-SMA staining denoted that expression, intensity and the number of immunoreactive cells in the livers with fatty change, fascioliasis, abscess and hydatid cyst were higher than in normal liver. Likewise, α-SMA immunopositive cells in livers with fascioliasis were higher than fatty change, abscess and hydatid cyst groups. It is noteworthy that α-SMA expression by myofibroblasts was present or increased in some areas of normal liver, liver with an abscess, and liver with a hydatid cyst, but in fascioliasis-affected liver, α-SMA expression was present throughout the liver and with higher intensity (Figure 4, α-SMA). On the other hand, immunolabelling results for β-catenin showed no or very minimal cellular expression in the normal liver. Compared to the normal liver, those affected by fascioliasis exhibited diffuse membranous expression in parenchymal cells and cytoplasmic membranous expression in bile duct cells. Also, we detected a nuclear

expression for β-catenin in the livers with fatty change while it was a membranous form in the livers affected by the abscess and hydatid cyst. Further, the immunoreactive cells for β-catenin were high in the liver groups with fascioliasis, fatty change, abscess and hydatid cyst respectively. However, the expression of this marker in all the affected liver groups was higher than the normal one (Figure 4, β-catenin). Regarding CD68 expression changes, we demonstrated that no immunoreactive cell existed in the normal liver. Livers affected by fascioliasis, showed some CD68 immunopositive Kupffer cells in the liver parenchyma and in some macrophages in inflamed areas. Likewise, in the livers with fatty change and hydatid cyst, there was positive immunoreaction in a few Kupffer cells in the liver parenchyma against CD68. Also, the liver had an abscess, showed a few CD68-positive macrophages in the inflammatory site and no positive immunoreaction in parenchymal Kupffer cells (Figure 5, CD68). Our comparative immunohistochemical results for Ki67 showed moderate immunopositivity in the normal liver. However intense positive nuclear immunoreaction was observed in some hepatocytes in the liver parenchyma of the fascioliasis group. So positive nuclear Ki67 immunoreaction in a higher number of cells in inflammatory sites indicates a higher tendency for proliferation in these cells. However, in the other liver groups including fatty change, hydatid cyst and abscess, we observed Ki67-positive immunoreaction in a few parenchymal cells (Figure 5, Ki67).

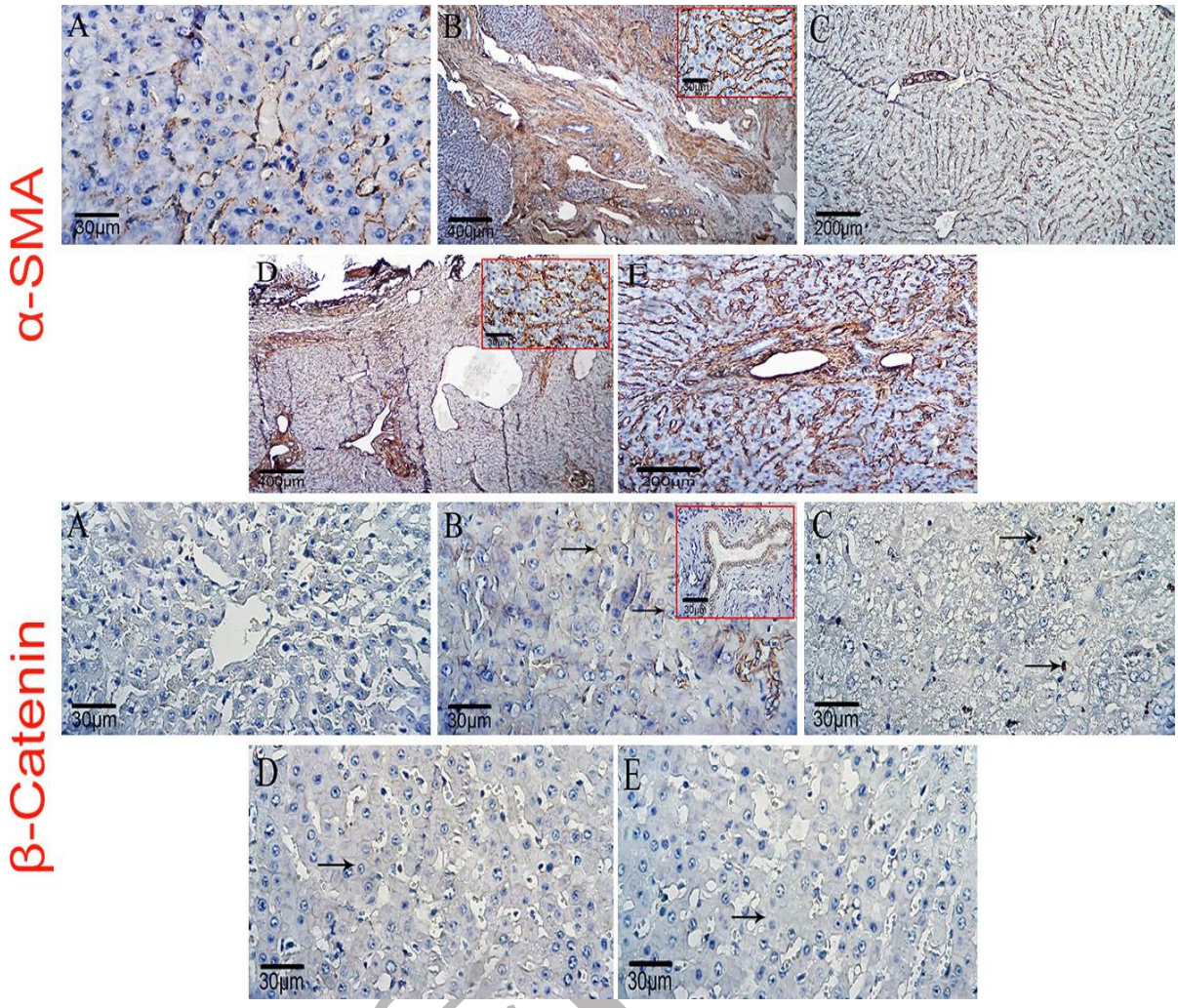

Figure 4: Comparison of α-SMA and β-catenin expression changes in common bovine liver lesions leading to fibrosis. (A) Normal liver with low α-SMA protein expression by myofibroblasts around hepatocytes in sinusoidal spaces. (B) Liver affected by fascioliasis with intense α-SMA expression by myofibroblasts around hepatocytes and in fibrotic areas of the liver (brown staining). (C) Hepatic fatty change with α-SMA expression. (D) Immunolabeling of the liver with an abscess, exhibiting α-SMA expression. (E) α-SMA expression in myofibroblasts in the liver with a hydatid cyst. (IHC, α-SMA). (A) The normal liver shows no or very minimal β-catenin expression in its cells. (B) Liver affected by fascioliasis exhibiting diffuse membranous expression in parenchymal cells (arrow) and cytoplasmic membranous expression in bile duct cells (arrow in the small inset). (C) Liver with fatty change, displaying nuclear β-catenin expression in some hepatocytes (arrow). (D) Liver with an abscess, showing

membranous β-catenin expression (arrow). (E) Liver with a hydatid cyst exhibiting very low membranous β-catenin expression in parenchymal cells (arrow). (IHC, β-catenin).

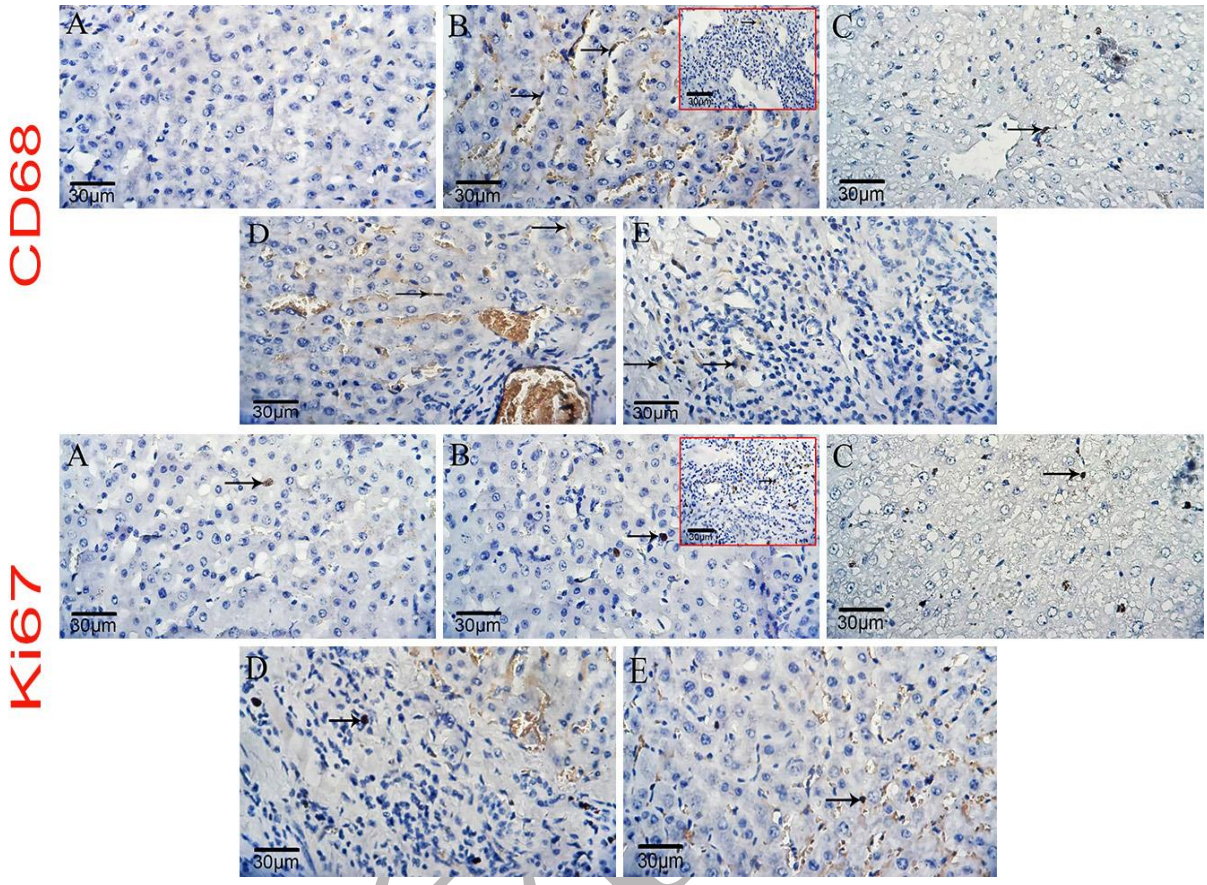

Figure 5: Changes in CD68 and Ki67 expression in parenchymal cells of bovine livers with common lesions leading to fibrosis. (A) Normal liver without CD68 expression in parenchymal (Kupffer) cells. (B) The liver affected by fascioliasis, with CD68 immunopositivity of Kupffer cells (arrow). (C) Hepatic fatty change with positive immunoreactive Kupffer cells against CD68 (arrow). (D) The liver affected by a hydatid cyst with CD68-positive immunoreaction of a few Kupffer cells (arrow). (E) The liver has an abscess and a few CD68-positive cells (arrow). (IHC, CD68). (A) Normal liver with a small number of hepatocytes exhibiting moderate positive nuclear immunoreaction (arrow) against Ki67. (B) Liver affected by fascioliasis with intense Ki67-positive immunoreactivity (arrow). (C) Liver with fatty change and positive nuclear immunoreaction against Ki67 in a few parenchymal liver cells. (D) The liver has an

abscess with immunopositivity for Ki67 in a few immune cells (arrow). (E) Liver with a hydatid cyst and Ki67 immunopositivity in a few parenchymal cells (arrow). (IHC, Ki67).

4. Discussion

The results of the present study showed that, according to Table 1, severe fatty liver (fatty liver grade III) and grade III cholangiohepatitis caused by Fasciola were more prevalent in the age group above 4.5 years compared to other age groups.

The enzymatic evaluation results of this study indicate a significant increase in blood ALT levels in cattle with liver fascioliasis compared to normal liver and other lesions ($P<0.05$). Additionally, compared to other groups, a significant increase in blood AST and GGT levels was observed in cattle with fascioliasis and severe fatty liver ($P<0.05$). The rise in serum AST activity is a well-documented indicator of liver disease. This enzyme increase has been observed in cattle with fatty liver, cirrhosis, and fascioliasis (13), which is consistent with the findings of this study. It is noteworthy that the AST test has high sensitivity and specificity for liver disease associated with severe damage. Additionally, an elevation in GGT has been reported in both natural and experimental mycotoxicosis cases and cases of bovine liver fascioliasis (13). In this study, an increase in both AST and GGT enzymes was observed not only in liver fascioliasis, which has been previously reported but also in fatty livers. The results of the present study demonstrated that among common bovine liver lesions, including fascioliasis, fatty liver change (hepatic steatosis), abscess, and hydatid cyst (cystic echinococcosis), the expression of β-catenin and αSMA markers was significantly increased ($P<0.05$) in fascioliasis lesions compared to normal liver and other lesions. Regarding the αSMA antibody, the present study indicated its lack of expression in normal liver, while its expression increased with the severity of fibrotic lesions. Specifically, in fascioliasis, αSMA expression was elevated in both parenchymal and perisinusoidal areas and fibrotic and portal

regions, observed in hepatic stellate cells. Various and controversial reports have been presented concerning the level of α-SMA expression in quiescent (stellate) hepatic cells of normal human and animal livers. For instance, a recent study concerning hepatic fibrosis as a common pathological change in dairy cattle with fatty liver showed immunohistochemical staining for α-SMA in normal livers and those with moderate and severe fatty livers. This immunohistochemical expression indicated an increase in hepatic stellate cells number and their staining intensity in severe fatty livers compared to moderate fatty and normal livers (14). In contrast, another study reported the lack of α-SMA expression for bovine hepatic stellate cells. Although α-SMA serves as a marker for activated myofibroblasts, it has been concluded that the lipid content in stellate cells may influence their morphology and function (15). Furthermore, another study described that in the normal livers of some domestic ruminants, including cattle and goats, as well as four wild ruminant species in a zoo, stellate cells exhibited positive reactions with desmin and vimentin antibodies despite being negative for α-SMA. Kupffer cells only showed a positive reaction with lysozyme. Additionally, both stellate and Kupffer cells demonstrated a specific distribution within the acinar lobular structure of the liver (16). Therefore, considering the conditions and type of α-SMA immunohistochemical staining in the present study, the obtained results for α-SMA conflict with the findings of Carollo et al. (2012) and Uetsuka et al. (2007) but are consistent with the results of Zhang et al. (2023). Regarding the localized pattern of α-SMA expression in different regions of the liver parenchyma, the results from various studies are generally similar, with α-SMA expression being more prominent in perisinusoidal areas compared to periportal and pericentral regions (17). However, in the study on bovine liver, considering that positive α-SMA expression has only been reported in one study (14), the present study also observed the localization of this protein expression in perisinusoidal areas of the bovine liver, similar to human liver. Overall, in the present study, the results regarding the localization of α-SMA expression in the liver are

consistent with the previous studies. However, it should be noted that in the current investigation, in addition to the perisinusoidal expression of α-SMA in common bovine liver lesions, α-SMA expression also increased in fascioliasis lesions with increased fibrosis in periportal regions. Furthermore, considering the Figure 4E, the level of α-SMA expression in livers affected by hydatid cyst (cystic echinococcosis) also increased in periportal areas and around central veins. It can be suggested that in bovine liver lesions, depending on the type and morphology of the lesions, α-SMA expression in hepatic stellate cells may vary in different regions, such as perisinusoidal, pericentrally around the central vein and periportal areas, although it is primarily perisinusoidal. The results of another study indicate that stellate cells may be responsible for the synthesis of type I collagen in the development of parasitic fibrosis caused by cystic echinococcosis in the bovine liver (18). In the present study, an increase in stellate cells (myofibroblasts) with elevated α-SMA expression was also observed around hydatid cysts with increased connective tissue, which is in line with the aforementioned study. There are diverse and controversial views regarding the expression and localization of β-catenin in the human liver. It is stated that in the normal liver, β-catenin is localized to the membrane, and the Wnt/β-catenin pathway is activated in pericentral hepatocytes (19). Additionally, β-catenin is reported to be expressed throughout the adult human liver, with this protein observed at the cell surface across the liver lobule, although it exhibits cytoplasmic and nuclear localization in pericentral cells. Consequently, in the normal adult liver, β-catenin signaling is consistently active in pericentral hepatocytes within the lobule (20). However, the results of a study on β-catenin expression in liver fibrosis demonstrated that β-catenin is not expressed in normal human hepatocytes, while the majority of β-catenin expression was observed in high-grade fibrotic liver tissues (21). Conversely, results of another research reported that β-catenin is primarily expressed in the cytoplasmic membrane of hepatocytes and normal bile ducts (22). Overall, considering the results of human studies on β-catenin expression in liver tissue, it can

be concluded that there is no consensus regarding the expression of this marker in normal liver. Regarding β-catenin expression in bovine liver, no study has been conducted thus far. However, several studies have been performed on mouse liver, and some of their results are mentioned here. In a study, it was stated that both in normal and diseased livers, β-catenin expression was present in a membranous pattern. Nevertheless, sinusoidal localization of β-catenin in the control group was observed in pericentral hepatocytes, but it was absent in the portal space. The study concluded that hepatocytes, cholangiocytes, and macrophages are not a source of the zonal regulation of Wnt, but rather, Kupffer cells serve as the major source of Wnt for the zonal regulation of β-catenin activation during liver regeneration (23). Another study reported a significant increase in the expression of total and active cytoplasmic β-catenin in normal and treated male rat livers and suggested that the Wnt/β-catenin pathway plays a crucial role in the activation and normal proliferation of adult rat hepatic stem cells (24). However, it is noteworthy that no study has been conducted regarding β-catenin expression in bovine liver lesions, and the present study is the first to demonstrate that, unlike human and murine livers, β-catenin expression was not observed in normal bovine liver. Nevertheless, a significant elevation in this protein expression was observed with an increase in the severity of fibrotic lesions, particularly in fascioliasis. It is worth mentioning that nuclear localization of this protein was detected in the fatty bovine livers instead of membranous expression. On the other hand, in livers affected by fascioliasis, in addition to the membranous expression of β-catenin in parenchymal cells, cytoplasmic membranous expression was also observed in inflamed or hyperplastic bile ducts, whereas this protein was not expressed in bile ducts of normal liver, fatty liver, or livers with hydatid cysts. CD68 is one of the specific markers for identifying Kupffer cells in the liver and macrophages. The results of the present study indicated a relative increase in CD68-positive cells in livers with lesions, but the number of these cells was markedly higher in fascioliasis compared to other lesions. Notably, firstly, CD68 expression in

bovine liver was primarily perisinusoidal, and secondly, in livers affected by fascioliasis and abscess, this marker was also expressed in some inflammatory cells (macrophages) within inflammatory foci. In a study, the results of CD68 immunohistochemical staining in fatty and normal livers showed that elongated, spindle-shaped Kupffer cells were diffusely present along sinusoids throughout the hepatic lobules (25). In the present study, Kupffer cells were also observed in a perisinusoidal and diffuse distribution in the livers of cattle with common lesions. However, CD68 expression was negative in normal liver. A study has been conducted to evaluate Kupffer cells (CD68 and Lysozyme) in diethylnitrosamine-induced hepatocellular carcinomas in monkeys. The findings denote that the reduction or loss of Kupffer cells in hepatocellular carcinoma and the surrounding parenchyma may result from the capillarization of hepatic sinusoids, which occurs during the processes of cirrhosis and carcinogenesis (26). In contrast to the aforementioned study, the present investigation observed a relative increase in CD68-positive Kupffer cells in liver lesions, particularly fascioliasis. The crosstalk between stellate cells and Kupffer cells plays a decisive role in the development of liver fibrosis. Macrophages produce various mediators that activate stellate cells. These fibrogenic mediators derived from macrophages include TNFα, IL-1β, Oncostatin M (OSM), PDGF, and TGFβ. The macrophage-derived factors responsible for activating stellate cells and promoting fibrosis progression include TGF-β and IL-13 (27). Numerous studies have demonstrated that reducing the release of cytokines and the infiltration of inflammatory cells (such as macrophages) can prevent and even reverse liver fibrosis (28). On the other hand, it has been established that mutations involving the β-catenin and AXIN1/2 genes lead to inappropriate and sustained activation of the Wnt/β-catenin pathway, thereby disrupting the regulation of various cellular functions, such as proliferation, apoptosis, and cell motility (29). Finally, regarding the proliferation marker Ki67, the results of the present study showed that although the number of cells with Ki67-positive nuclei was higher in liver lesions compared to normal liver, the increase

in different lesions relative to each other was not significant (P<0.05). It seems that this relative increase in Ki67-positive cells suggests that the regeneration of hepatocytes may be activated with the exacerbation of liver injury (30). The results of the present study demonstrated that among common liver lesions leading to fibrosis in cattle observed at the slaughterhouse, fascioliasis exhibited the highest expression levels of α-SMA and β-catenin proteins compared to other lesions, including fatty change (fatty liver), abscess, and hydatid cyst. Additionally, considering the limited research conducted on these markers in bovine liver, the current study's findings indicate differences in the expression patterns of these proteins in bovine liver compared to human or murine liver. Specifically, in bovine liver and fibrotic lesions, all three patterns (perisinusoidal, periportal, and pericentrally around the central vein) of α-SMA expression were observed. Furthermore, in contrast to humans and mice, where nuclear expression of β-catenin has only been reported in hepatocellular carcinoma cases, nuclear localization of β-catenin was observed in parenchymal hepatocytes in fatty liver change. Moreover, unlike the human liver, where β-catenin expression is present in bile duct cells under normal conditions, no β-catenin expression was detected in either parenchymal or bile duct cells of normal bovine liver, and only cytoplasmic membranous expression of β-catenin was observed in bile duct cells of livers affected by fascioliasis. Additionally, the results revealed a relative increase in the number of CD68-positive Kupffer cells in fascioliasis compared to other lesions, although their expression was not observed in normal liver. The evaluation of the proliferation marker Ki67 also demonstrated a relative increase in positive nuclear immunoreaction in some parenchymal cells of affected livers, although the differences were not statistically significant.

Overall, the findings of this study indicated that with increasing severity of fibrosis, the expression of CD68, β-catenin, α-SMA, and Ki67 markers also increases. In other words, with the initiation and progression of inflammation in the bovine liver, the concurrent activation of

stellate cells and Kupffer cells occurs. This event leads to the production of various cytokines and, particularly, intermediate filaments of the extracellular matrix, such as collagen and fibronectin, contributing to the regeneration of the damaged liver with connective tissue, ultimately resulting in liver fibrosis.

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Author's Contribution

PK: sampling and laboratory analyses, statistical analyses and interpretation of data as well as drafting the article; AA: Conception and design of the study, analysis and interpretation of data, final approval of the version to be submitted, Study design, participating in histopathological and immunohistochemical analyses, drafting the article or revising it critically for important intellectual content.

Ethics

This study was approved by the Research Ethics Committees of the Islamic Azad University, Urmia Branch, with the approval number of IR.IAU.URMIA.REC.1402.010 on April 16, 2023.

Conflict of Interest

There is no conflict of interest between the authors.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

5. References

1. Mann DA, Marra F. Fibrogenic signalling in hepatic stellate cells. J Hepatol. 2010;52(6):949–50.

2. Chen Y, Chen X, Ji YR, Zhu S, Bu FT, Du XS, et al. PLK1 regulates hepatic stellate cell activation and liver fibrosis through Wnt/β-catenin signalling pathway. J Cell Mol Med. 2020;24(13):7405–16.

3. Duspara K, Bojanic K, Pejic JI, Kuna L, Kolaric TO, Nincevic V, et al. Targeting the Wnt signaling pathway in liver fibrosis for drug options: an update. J Clin Transl Hepatol. 2021;9(6):960–71.

4. Nishikawa K, Osawa Y, Kimura K. Wnt / β -Catenin signaling as a potential target for the treatment of liver cirrhosis using antifibrotic drugs. Int J Mol Sci. 2018;19(10):3103.

5. Foglia B, Cannito S, Bocca C, Parola M, Novo E. ERK pathway in activated, myofibroblast-like, hepatic stellate cells: A critical signaling crossroad sustaining liver fibrosis. Int J Mol Sci. 2019;20(11):2700.

6. Liu Y, Tian F, Shan J, Gao J, Li B, Lv J, et al. Kupffer Cells: Important Participant of Hepatic Alveolar Echinococcosis. Front Cell Infect Microbiol. 2020;10(January):1–9.

7. Ghalandarzadeh B, Amniattalab A. Prevalence and pathological evaluation of hepatic fatty change in cattle slaughtered at urmia abattoir, Northwest Iran. Iraqi J Vet Sci. 2019;33(1):45–50.

8. Stojević Z, Piršljin J, Milinković-Tur S, Zdelar-Tuk M, Beer Ljubić B. Activities of

AST, ALT and GGT in clinically healthy dairy cows during lactation and in the dry period. Vet Arh. 2005;75(1):67–73.

9. Jóźwik A, Strzałkowska N, Bagnicka E, Grzybek W, Krzyżewski J, Poławska E, et al. Relationship between milk yield, stage of lactation, and some blood serum metabolic parameters of dairy cows. Czech J Anim Sci. 2012;57(8):353–60.

10. Al-Mahmood S, Al-Sabaawy H. Fasciolosis: grading the histopathological lesions in naturally infected bovine liver in Mosul city. Iraqi J Vet Sci. 2019;33(2):379–87.

11. Kim S, Kim Y, Kim SE, Kim HJ. Comparison of biomarkers between hepatic tumors in rat models and a dog. In Vivo (Brooklyn). 2023;37(1):252–61.

12. Abou Monsef Y, Kutsal O. Immunohistochemical evaluation of hepatic progenitor cells in different types of feline liver diseases. J Vet Med Sci. 2021;83(4):613–21.

13. Moreira C., Souza S., Barini A., Araújo E., Fioravanti MC. Serum γ-glutamyltransferase activity as an indicator of chronic liver injury in cattle with no clinical signs. Arq Bras Med Veterinária e Zootec. 2012;64(6):1403–10.

14. Zhang C, Shao Q, Liu M, Wang X, Loor JJ, Jiang Q, et al. Liver fibrosis is a common pathological change in the liver of dairy cows with fatty liver. J Dairy Sci. 2023;106(4):2700–15.

15. Uetsuka K, Nishikawa S, Isobe K, Nakayama H. Histopathological characteristics of Kupffer cells and ito cells in the porcine and bovine liver. J Vet Med Sci. 2007;69(7):767–70.

16. Carollo V, Di Giancamillo A, Vitari F, Schneider R, Domeneghini C. Immunohistochemical aspects of Ito and Kupffer Cells in the liver of domesticated and wild ruminants. Open J Vet Med. 2012;02(03):129–36.

17. Akpolat N, Yahsi S, Godekmerdan A, Yalniz M, Demirbag K. The value of α-SMA in the evaluation of hepatic fibrosis severity in hepatitis B infection and cirrhosis development: A histopathological and immunohistochemical study. Histopathology. 2005;47(3):276–80.

18. Atmaca HT, Gazyagci AN, Terzi OS, Sumer T. Role of stellate cells in hepatic echinococcosis in cattle. J Parasit Dis. 2019;43(4):576–82.

19. Xu C, Xu Z, Zhang Y, Evert M, Calvisi DF, Chen X. β-Catenin signaling in hepatocellular carcinoma. J Clin Invest. 2022;132(4):e154515.

20. Monga SP. B-Catenin signaling and roles in liver homeostasis, injury, and tumorigenesis. Gastroenterology. 2015;148(7):1294–310.

21. Ge WS, Wang YJ, Wu JX, Fan JG, Chen YW, Zhu L. β-catenin is overexpressed in hepatic fibrosis and blockage of Wnt/β-catenin signaling inhibits hepatic stellate cell activation. Mol Med Rep. 2014;9(6):2145–51.

22. Yun KJ, Han WC, Choi SC, Kim TH. Immunohistochemical study of β-catenin expression between hepatocellular carcinoma and cholangiocarcinoma. Cancer Res Treat. 2002;34(2):117–21.

23. Yang J, Mowry LE, Nejak-bowen KN, Okabe H, Diegel CR, Lang RA, et al. Beta-Catenin signaling in murine liver zonation and regeneration: A Wnt-Wnt situation! Hepatology. 2014;60(3):964–76.

24. Apte U, Thompson MD, Cui S, Liu B, Cieply B, Monga SPS. Wnt/beta-catenin signaling mediates oval cell response in rodents. Hepatology. 2008;47(1):288–95.

25. Lefkowitch JH, Haythe JH, Regent N. Kupffer cell aggregation and perivenular distribution in steatohepatitis. Mod Pathol. 2002;15(7):699–704.

٥٢٦ 26. Lapis K, Zalatnai A, Timár F, Thorgeirsson UP. Quantitative evaluation of lysozyme-
٥٢٧ and cd68-positive kupffer cells in diethylnitrosamine-induced hepatocellular
٥٢٨ carcinomas in monkeys. Carcinogenesis. 1995;16(12):3083–5.

٥٢٩ 27. Matsuda M, Seki E. Hepatic stellate cell-macrophage crosstalk in liver fibrosis and
٥٣٠ carcinogenesis. Semin Liver Dis. 2020;40(3):307–20.

٥٣١ 28. Zhang C, Liu XQ, Sun HN, Meng XM, Bao YW, Zhang HP, et al. Octreotide
٥٣٢ attenuates hepatic fibrosis and hepatic stellate cells proliferation and activation by
٥٣٣ inhibiting Wnt/β-catenin signaling pathway, c-Myc and cyclin D1. Int
٥٣٤ Immunopharmacol. 2018;63(May):183–90.

٥٣٥ 29. Khalaf AM, Fuentes D, Morshid AI, Burke MR, Kaseb AO, Hassan M, et al. Role of
٥٣٦ Wnt/β-catenin signaling in hepatocellular carcinoma, pathogenesis, and clinical
٥٣٧ significance. J Hepatocell Carcinoma. 2018;5:61–73.

٥٣٨ 30. Kimura M, Nishikawa K, Osawa Y, Imamura J. Inhibition of CBP / β - - catenin
٥٣٩ signaling ameliorated fibrosis in cholestatic liver disease. Hepatol Commun.
٥٤٠ 2022;6:2732–47.

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