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Ovine Hepatic *Echinococcus granulosus* Infection Induces Histopathological Alterations and Immune Responses

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Abstract **Background:** The parasite *Echinococcus granulosus* is still endemic in many nations worldwide, particularly in developing nations. The main organ where the parasite infests is the liver. is a significant zoonotic infection that mostly affects endemic areas and impacts millions of people globally. Techniques. A granulomatous tissue reaction is caused by an ongoing infection with an *E. granulosus* hydatid cyst, progressively establishing an immunological milieu marked by the buildup of monocytic and lymphocyte cells. IL-10-producing CD8+ T cells and CD4+ T-cell-mediated cellular immune responses are essential during the establishment phase of secondary *E. granulosus* s.s. infection. **Methods:** Liver samples (40 infected liver and 20 non-infected) from animals infected with hydatid cysts were collected from licensed governmental butchers and slaughterhouses located in local markets within the Diyala Governorate; no animals were harmed for this study, as the samples were from previously sacrificed animals. **Results:** The study examined clinical and histological alterations in liver tissue sections, with a focus on the impact of hydatid., The results of histological examination of liver sections showed that the infected livers contained increased sinusoid and central vein dilation; portal and central vein congestion; necrosis of hepatic tissue; atrophy; and increased inflammatory cell infiltration. compared to the noninfected livers, which had no histologic lesions. The current work involves assessing the expression of PD-L1, CD4, CD8 proteins in the liver infected with *E. granulosus*. **Conclusion:** In addition to increased inflammatory cell infiltration in the liver, a hydatid cyst infection results in a variety of clinical and biochemical alterations. These results open the door for further studies focused on early detection, prevention and focused treatment approaches for this common illness.

Key Words *Echinococcus granulosus*, Hepatic Hydatid Cyst, CD4, CD8, PDL1, Ovine Hydatidosis, Histopathological Alterations, Host Immune Response, Cytokine Expression, Liver Pathology, Parasitic Zoonosis

INTRODUCTION

Hydatid cysts disease, caused by the larval stage of cestode worm (*Echinococcus granulosus*), is a considerable zoonotic infection that affects millions of people worldwide, essentially in endemic regions [1]. The liver is the most predominantly involved organ, as it accounting for approximately 50-70% of cases, followed by the lungs and other organs [2]. The worm's life cycle involves canines as final hosts and secondary hosts such as sheep, cattle and humans, where the larvae develop slow-growing hydatid cysts [3]. Chronicity and frequently asymptomatic evolution nature of hepatic hydatidosis complicates early diagnosis, result in progressive destruction of liver tissue and severe complications if left untreated [4].

Mechanical and immunological influences are involved in the pathogenesis of hepatic cysts. As the cyst enlarges, it compresses surrounding parenchyma, leading to atrophy, fibrosis and portal hypertension in advanced cases [5]. Additionally, the host immune response triggers a peri-cystic inflammatory reaction, characterized by granuloma formation and fibrotic encapsulation [6]. The influence of hepatic hydatid cysts on liver function varies according to the size, number and location of the cysts. It can disrupt the hepatic architecture and normal bile flow causing cholestasis or secondary sclerosing cholangitis [7], especially in the presence of large or multiple cysts. In addition, long-standing inflammation may result in oxidative stress and hepatocyte damage, as supported by the elevation of liver enzymes and derangement in synthetic function in a few patients [8]. The dynamic interaction between the host and the parasite, which leads to histological changes in the liver tissue surrounding the hydatid cysts, leads to marked structural and inflammatory changes. Mechanical stretching of the cyst also stimulates pericyst fibrosis, forming a dense

collagenous layer (lamellar outer sheath) separating the parasite from the host liver tissue [9]. Microscopically, the area surrounding the cyst shows chronic granulomatous inflammation, with infiltrates of numerous defense cells including lymphocytes, plasma cells, eosinophils and epithelioid macrophages [10], the liver and other organs [2].

In the process of controlling parasitic infection, the immune response plays fundamental role, with T lymphocytes, in particular CD4+ and CD8+ T cells, being essential to both protective immunity and immunopathology [11]. CD4+ T cells, firstly., through their Th1 and Th2 cytokine patterns, regulate the immune response against hydatid cysts. Th1 responses, featured by IFN-γ and IL-2 production, are associated with parasite inclusion, while Th2 responses (IL-4, IL-10) may participate to chronic infection by inhibiting effective immunity [12]. Conversely, CD8+ cytotoxic T cells are contributed in direct parasite killing through mediated cytotoxicity of perforin/granzyme-and Fas-FasL interactions [13]. In liver hydatidosis, the strategies of modulated the immunity of *E. granulosus* often lead to response of both Th1/Th2 response, which may determine cyst viability and clinical outcomes [14]. At the immunobiological level, the host immune response to the cestode *Echinococcus granulosus* involves a complex interaction of Th1/Th2 cytokine polarization and immune evasion mechanisms of parasite. Initially, the response mediated by Th1cytokines (IFN-γ, IL-12) predominate, to promote macrophage activation and granuloma formation in an attempt to contain the parasite [15]. in contrast, the parasite counteracts this by stimulating a Th2-skewed response (IL-4, IL-10, IL-13), which inhibits cytotoxic immunity response and facilitates chronic infection [13]. The glycoprotein contents of cyst's laminated layer (e.g., antigen B) that modify the function of dendritic cells and suppress neutrophil recruitment, contributing to immune tolerance [16]. Additionally, an upregulation of regulatory T cells (Tregs) in chronic infections, further demoralises effective immune clearance [14]. These immunological adaptations permit the cyst to persist while causing progressive pericystic liver damage, leading to the formation of fibrosis [14]. Recently, it was reported that PD-L1 (Programmed Death-Ligand 1) is upregulated by *E. granulosus* to inhibit host T-cell responses. The hydatid cyst induces PD-L1 expression on macrophages and dendritic cells of the host within the pericystic granuloma, elevating T-cell exhaustion [17]. Moghaddam *et al.* [17] mention that PD-L1 binds to PD-1 on CD4+ and CD8+ T cells, inhibiting their activation and cytokine production (e.g., IFN-γ), thereby facilitating chronic infection. McManus *et al.* [2] pointed out that High PD-L1 levels correlate with cyst viability and recurrence post-surgery. Understanding of the mechanisms underlying liver injury in hydatid disease is essential for the optimal therapeutic intervention, i.e., surgical resection, percutaneous drainage and/or antiparasitic therapy [5].

Objectives of the Study

The objective of the present study was to assess foam changes and immune response in liver due to *Echinococcus granulosus* infection in sheep.

Primary Objective

To evaluate the histopathological changes of liver in infected sheep, such as structural distortion, inflammatory cell infiltration and tissue degeneration and fibrosis.

Secondary Objectives

- The immunological reaction on infected liver tissues was assessed with the $CD4^+$ T lymphocytes expression
- To evaluate the role of CD8(+) cytotoxic T cells during the local immune response
- Investigation the expression of PD-L1 as an immunoregulatory marker associated with anti-immune mechanism
- To associate histopathological changes with expression of immunologic markers in infected liver tissue

METHODS

Histopathological Examination for Liver Sample

The use of animals in this study was authorised by the College of Education for pure sciences research ethics committed the (approval serial number: CEPEC/01), University of Diyala. Liver samples (40 infected livers and 20 non-infected livers) from animals infected with hydatid cysts were collected from licensed governmental butchers and slaughterhouses located in local markets within the Diyala Governorate; no animals were harmed for the purposes of this study, as the samples were from previously sacrificed animals.

Before beginning the histological study, the livers affected by hydatid cysts were washed with distilled water and wiped with 70% ethanol. Then, a portion of the hydatid cyst fluid was withdrawn using a sterile needle for the purpose of diagnosis and to confirm the fertility of the cyst. Eosin stain was used for microscopic examination to confirm the viability of protoscolex isolated from liquid hydatid cyst

The samples were promptly transported to the laboratory and preserved in fixative solutions (10% neutral buffered formalin for a duration of 24 hours). To identify the histological changes caused by the hydatid cysts, traditional histology techniques with hematoxylin-eosin stains were utilized. A light microscope equipped with a camera was employed to capture images of the tissue sections at various magnifications. Additionally, PAS and Masson's staining techniques were applied.

Immunohistochemical (IHC) Tests

The paraffin blocks were cut into sections of 3 µm using a microtome (Thermo Fisher Scientific, United States), deparaffinized in an oven set at 68°C and then rehydrated through a series of alcohol concentrations. Manual immunohistochemical staining was carried out with kits from Thermo Fisher Scientific, United States. The components of this kit create a labeled streptavidin-biotin immunoenzymatically system for recognizing antigens. This technique involves a sequential incubation of the sample with a primary antibody that is not conjugated and specifically targets the antigen of interest. The secondary antibody, which is biotinylated, interacts with the primary antibody alongside the streptavidin bound to an enzyme and

the substrate-chromogen. Analysis of liver samples was conducted for CD4 helper T lymphocytes (Monoclonal Mouse Anti-Human CD4, DAKO, Denmark) and CD8 cytotoxic T lymphocytes (Thermo Fisher Scientific, USA) to identify T cells and PDL-1.

A grading of hepatocellular damage was performed based on the degeneration of epithelial cells, along with necrosis, vascular congestion, hemorrhage and inflammatory cell infiltration. The parameters mentioned were classified and quantified as mild, moderate and severe, as defined in Hassanein *et al.* [18]. Grading and scoring were carried out under 40x magnification to examine the inflammatory cells with stained cytoplasm, selecting approximately ten random microscopic fields for each evaluation [19]. The expression levels of CD4, CD8 and PDL-1 were assessed using immunohistochemistry with a Mouse Monoclonal antibody. Following Dako's instructions, a protocol was implemented to stain the tissue with Anti-CD4, CD8 and PDL-1 antibodies. Sections from the positive immunohistochemical scoring system were applied and a negative control was included.

RESULTS

Histological Changes of the Liver Affected by Hydatid Cysts

The histological examination of liver sections revealed significant differences between infected and noninfected livers. Infected livers exhibited increased sinusoid and central vein dilation, portal and central vein congestion, hepatic tissue necrosis, atrophy and heightened inflammatory cell infiltration. In contrast, noninfected livers showed no histologic lesions. Histochemical stains indicated that Masson's reaction was present in noninfected livers, with minimal fiber, while infected livers demonstrated a marked increase in fiber in the portal areas. There was a notable deposition of collagen fibers in the fibrous adventitial layer of the cyst and between hepatocytes. Periodic acid-Schiff staining further confirmed that infected livers had increased sinusoid and central vein dilation alongside necrosis and atrophy, unlike the noninfected counterparts (Figure 1-6).

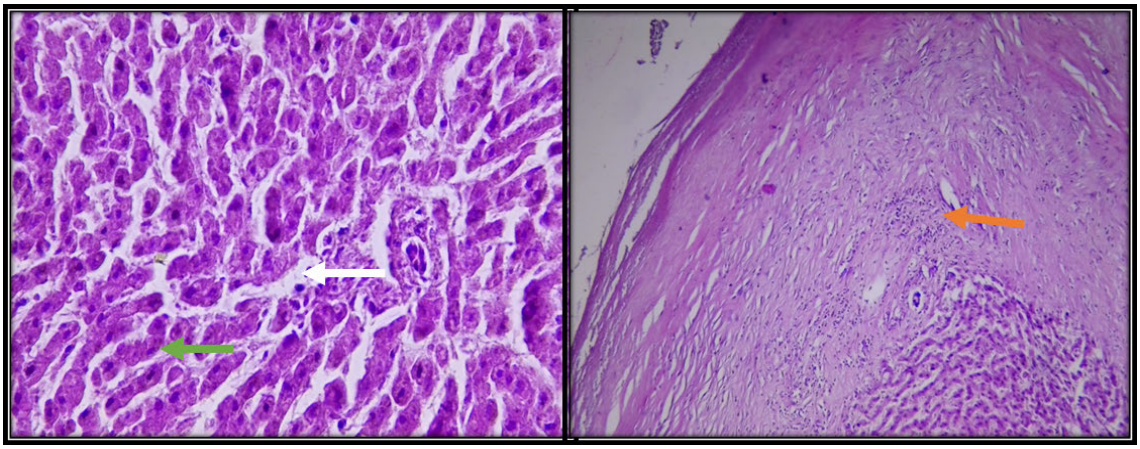

Figure 1(a-b): (a) A-Section in Normal Liver Sheep Reveals Sinusoids (Arrow White) and Hepatocytes Organized in Thread (Arrow Green) (H&E10X) and B-Section of an Infected Liver Demonstrates the Obvious Presence of a Significant Infiltration of Inflammatory Cells (Arrow Red) (H&E10X)

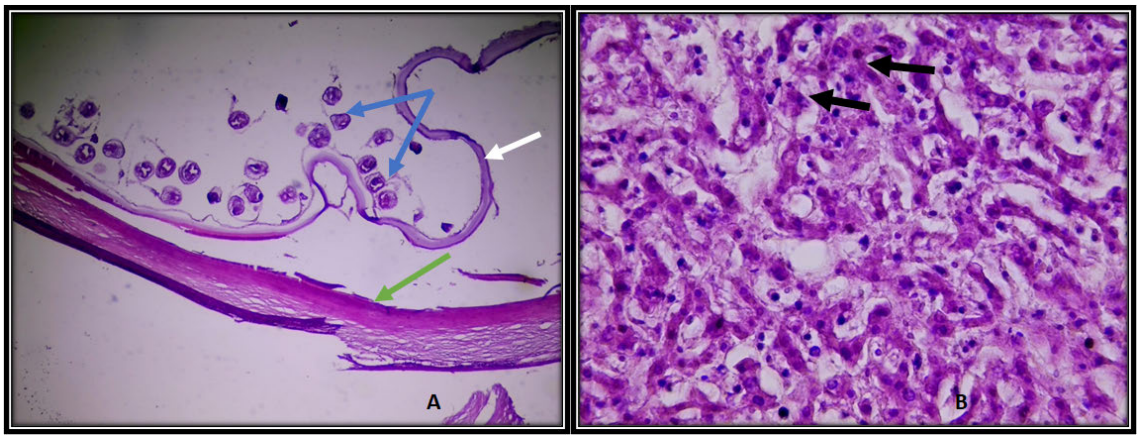

Figure 2(a-b): (a) An A-Section of an Infected Liver Reveals the Presence of the Liver's Wall of Septa (arrow green), Which Attaches to the Hydidt Cyst Wall (Arrow White) and the Presence of Protoscolex (Arrow Blue) in the Livers of Sheep that were Investigated (H&E 10X) and (b) B-Section of an Infected Liver Sheep Reveals a High Degree of Hepatocyte Necrosis (Arrow Black) (H&E40X)

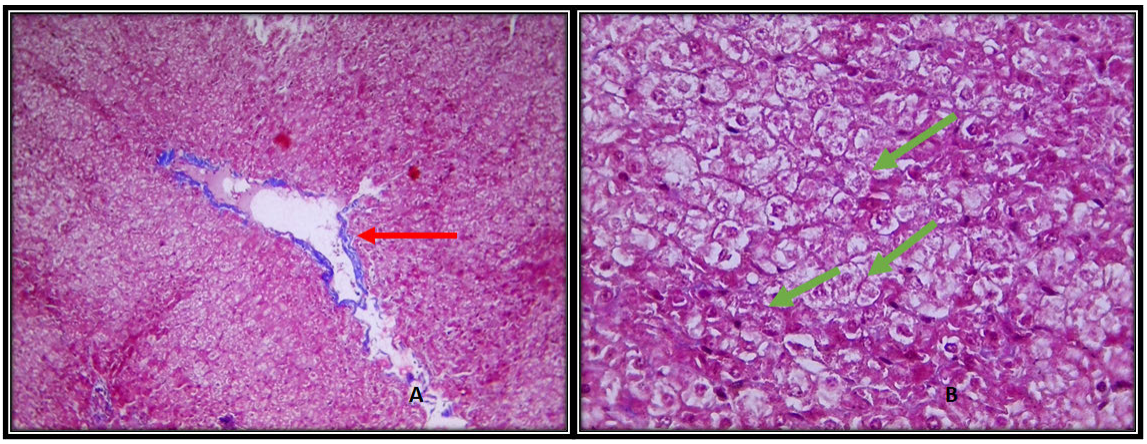

Figure 3(a-b): (a) A-Section in Normal of Liver Sheep Showing Collagen Deposition Only in the portal Area (Arrow Red).(Masson's Trichrome10x) and (b) B-Section in Normal of Liver Sheep Showe the Hepatocyte (Arrowgreen) (Masson'strichrome10x)

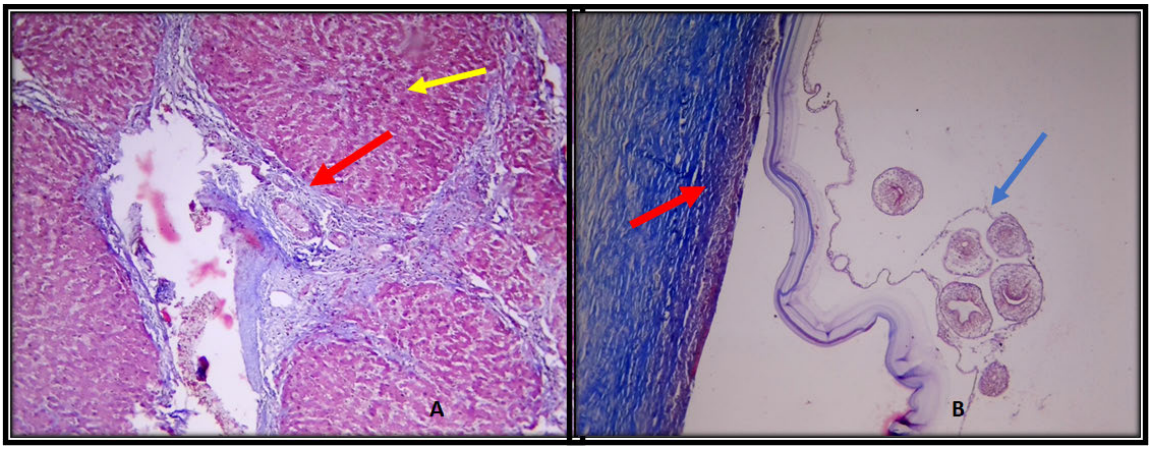

Figure 4(a-b): (a) Fibrosis (Arrow Red) and an Increase in Connective Tissue Surrounding the Portal and central Veins (Nodule) (Arrow Yellow) are Shown in the A-Section of Liver-Infected Sheep (40X, Masson'strichrome) amd (b) B-Section of Liver-Infected Sheep Demonstrates Protoscolex (Arrow Blue), Fibrosis of the Liver Wall (Arrow Red) and a Hydatid Cyst Wall (Arrow White) (Masson's trichrome 10X)

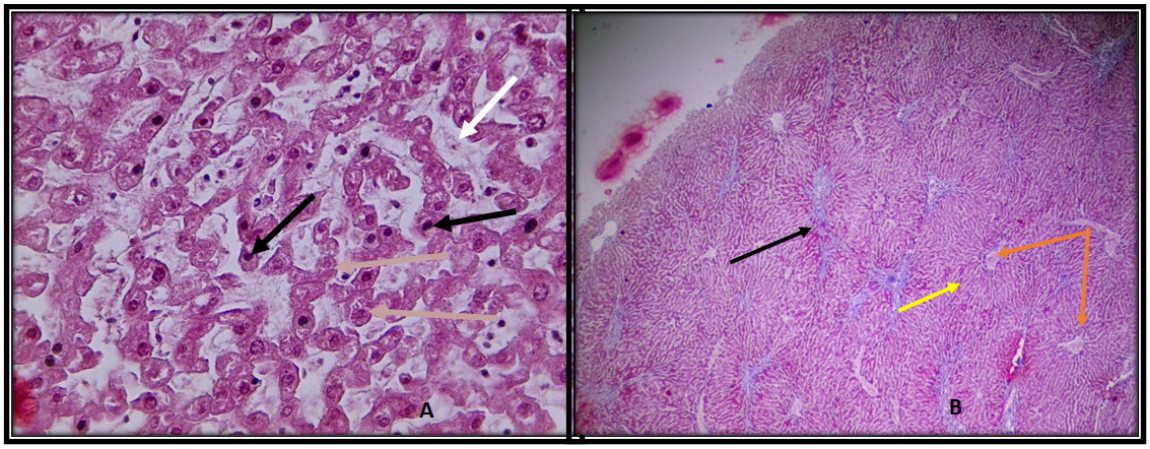

Figure 5(a-b): (a) A-Section of an Infected Sheep's Liver Demonstrates Atrophy in the Hepatocytes (Arrow Pink), Enlargement in the Sinusoids (Arrow White) and High Necrosis (Arrow Black) (Masson's Trichrome 10X) and (b) B-Section of Liver-Infected Sheep Exhibit Fibrosis (Arrow Red), an Increase in Connective Tissue Surrounding the Portal and Central Veins (Arrow Yellow) and the Enlargement and Congestion in the Central Veins (Arrow Brown) (The 4x Masson's Trichrome)

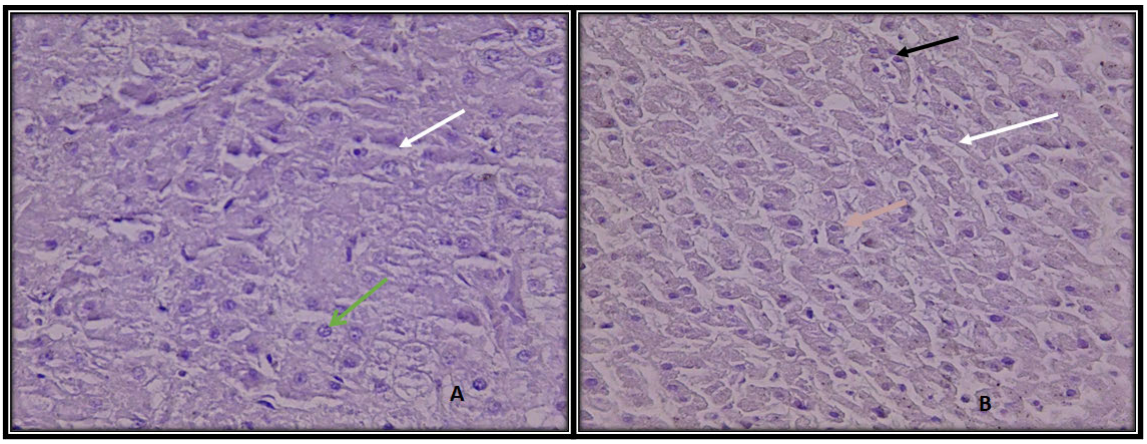

Figure 6(a-b): (a) A-Section of a Sheep in Normal Condition Displays the Sinusoids (Arrow White) and the Hepatocyte (Arrow Green) (PAS10X). High Necrosis (Arrow Black), Sinusoidal Expansion (Arrow White) and (b) Hepatocyte Atrophy (Arrow Pink) are all Visible in the B-section of an infected liver sheep (PAS10X)

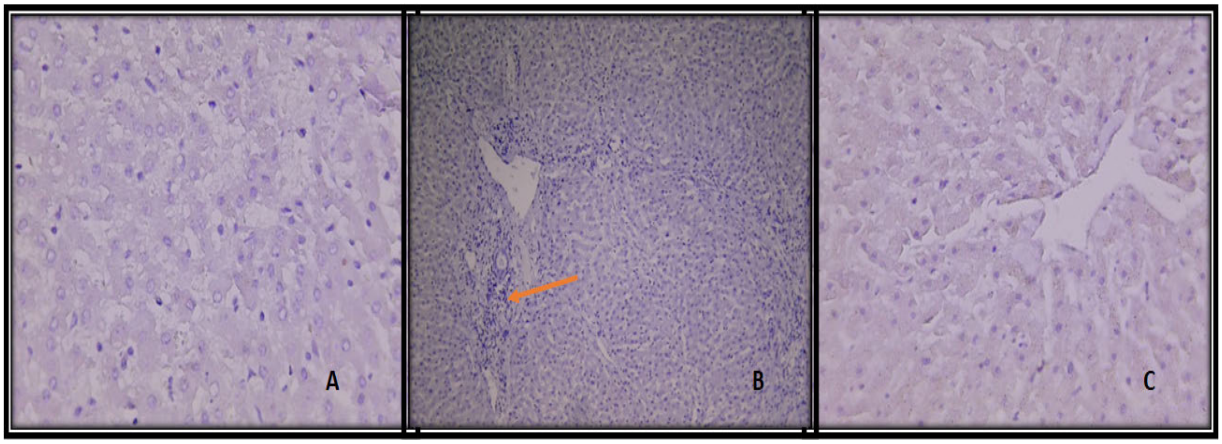

Figure 7(a-c): (a) A-Immunohistochemical Analyses PD-1 Protein Expression in Normal Liver in Study Groups Control Negative Group, (b) B-PD-L1 Expression in Liver Tissue Infected Group Showing Non Expression of PD-1 Protein but Showing Increase Inflammation Cells (Arrow Brown) and C- PD-L1 Expression in Liver Tissue Infected Group Showing Middle Expression of PD-1 protein (Score1)

Immunohistochemical Evaluation of PDL1, CD4 and CD8 in Liver-Infected Hydatid Cyst

PDL1The current work involved an evaluation of PD-1/PD-L1 protein expression and CD8 and CD4 in liver tissue sections in sheep infected with hydatid cysts. The current work involved an evaluation of PD-1/PD-L1 protein expression, as well as CD8 and CD4, in liver tissue sections from sheep infected with hydatid cysts. The results showed that PD-1 protein expression in negative control (normal liver tissue) was non-expression, while it was moderate expression in the infected group (Figure 7-8).

The results showed that the expression of CD4 in the negative control group (normal liver tissue) was not expressed, while highly increased expression was observed in the infected group (Figure 9).

DISCUSSION

This study indicates that sheep infected with hydatid cysts undergo various histopathological changes that differ in intensity. The severity of pathology caused by hydatid cysts varies based on the affected organs, the quantity and size of the cysts, as well as their interaction with neighbourin tissues [20]. While hydatid cysts can develop anywhere within the body of intermediate hosts (both humans and animals), they are predominantly found in the liver, as the hepatic capillary network acts as the first capillary filter for hexacanth embryos in circulation [21,22]. These cysts progressively develop within this organ [23]. A persistent infection with an *E. granulosus* hydatid cyst leads to a granulomatous tissue reaction, gradually creating an immune environment characterised by the accumulation of lymphocytes and monocytic cells [24,25].

However, infections resulting from hydatid cysts cause various histological changes. The current study's findings show significant alterations in liver tissue, including infiltration of inflammatory cells, necrosis, degeneration of liver tissue, fibrosis surrounding portal veins, congestion of the portal vein, expansion of sinusoids and atrophy of

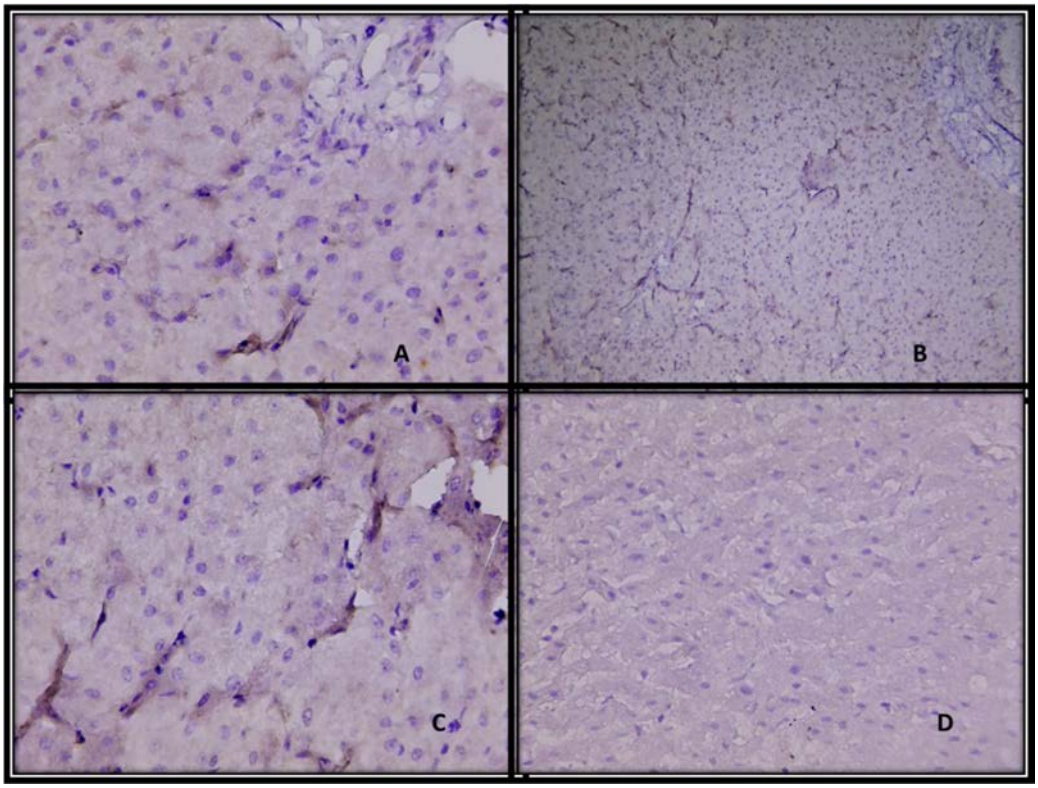

Figure 8(a-d): (a-c) CD8 Expression in Liver Tissue Infected Group Showing High Expression of CD8 (score2) and CD8 Expression in Normal Liver Tissue Group Showing no Expression of CD8

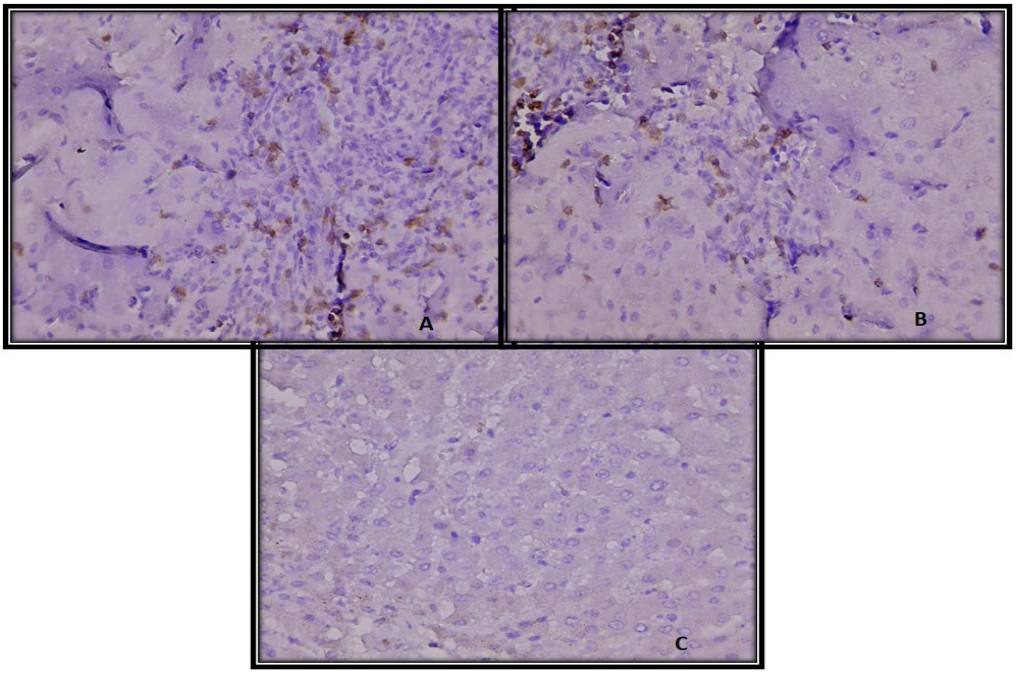

Figure 9(a-c): (a-b) CD4 Expression in Liver Tissue Infected Group Showing High Expression of CD4 (Score2) and (c) CD4 Expression in Normal Liver Tissue Group Showing Non Expression of CD4

hepatocytes, as show in Figure 2b, 5a-b and 6b. These observations agree with prior studies [26-28]. Similarly, numerous researchers have documented necrosis, fibrosis, dilatation of sinusoids, congestion of the portal vein and hepatocellular degeneration in the portal regions of sheep with infected livers [29-32]. These changes may be

attributed to the pressure and other space-occupying effects of the expanding cysts, leading to the replacement of host tissue and further degenerative changes in the affected organs. Recent studies have also shown that hepatic macrophages initiate adaptive immune responses and exacerbate fibrosis by releasing cytokines and chemokines and acting as specialized antigen-presenting cells [33,34]. Presenting and processing hydatid cyst antigens to other immune cells, such as T cells, which are also abundant in lesion sites, may be a significant role of hepatic macrophages [24]. Earlier research highlights the essential role of macrophages in liver fibrosis development, granuloma formation and inflammation following parasite infection [35,36]. Nonetheless, the precise mechanism through which macrophages actively contribute to the immune response during hydatid cyst infection is still not fully understood. Thus, the current study focused on investigating the characteristics and role of hepatic macrophages during the larval establishment and chronic infection of hydatid cyst. The present study revealed that inflammatory cells in sheep infiltrated variously around hydatid cyst lesions, with the highest inflammatory focus noted around the cysts (as in Figure 1b). This observation is consistent with Wang *et al.* [37]. In a mouse model of hydatid cyst. infection, the level of macrophage infiltration was correlated with the severity of liver fibrosis progression during infection (Figure 4a, b), indicating a potential role of macrophages in liver fibrosis, where heightened infiltration signifies the host's immune response to the parasites. When the parasite damages host tissue, vasoactive and chemotactic factors are released. These factors enhance blood vessel permeability and blood flow to the affected area, facilitating the migration of mast cells, eosinophils, basophils and macrophages from the bloodstream to the damaged site, thereby resulting in inflammation [29]. In reality, hydatid cysts' methods for reprogramming inflammatory cells are probably linked to the control of inflammation and/or immunological responses [38]. In line with earlier research [24]. In addition to causing maladjustment of the hepatic cords, the larger cysts exerted pressure on the hepatocytes and clogged the portal veins, which is consistent with [39]. These findings, together with those reported by AL-Azizzi *et al.* [21] support the current investigation, which found that fibrosis was more prevalent in cattle liver tissue than in sheep liver tissue. The fibrous tissue formed as the host's response to the inflammatory effect of the parasite, which was started in the early stages of the cyst's development. The severity of this response affects the development of the cyst; a strong reaction will cause the parasite to degenerate or die, whereas a moderate reaction permits the formation of a viable cyst [40].

Expansion in sinusoids occur in the liver in the present study (Figure 5a, b and 6b). where the size of the cysts and the pressure that the expanding cyst exerted were linked to these changes [41,42]. The infected livers showed greater collagen deposition in the portal region, surrounding the adventitial layer and between the hepatocytes. This suggests that the parasitized organ produced more collagen as a result of ongoing irritation from the growing cysts.

CONCLUSIONS

The chance of infection with hydatid cysts increases through dealing with and living in endemic areas and through direct contact with intermediate and final hosts. Through IHC evaluation, it was found that the parasite stimulates a protective inflammatory immune response represented by a high increase in the indicators PDL1, CD4 and CD8.

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