Research Article

Immunohistochemical and Histopathological Characterization of Immune Changes in the Host-tissue Reaction site of Murine Cystic Echinococcosis

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Abstract | Cystic echinococcosis (CE), a zoonotic disease that can affect humans and livestock, is caused by the larval stage of the *Echinococcus granulosus*. CE is more prevalent and is presently identified as a significant parasite that should be prevented and controlled globally. This work investigated the immune response and apoptosis expression in hepatic cystic echinococcosis. Experimentally hydatid cysts infection of BALB/c mice was established, and the manifestation of immune actions and apoptosis were detected histopathologically and by immunohistochemical markers staining. The infected livers with hydatid cysts from the mice were processed for routine paraffin block technique. The sections of infected livers that were embedded in paraffin underwent immunohistochemistry. Several immunolabeled liver sections with anti-CD3, CD4, CD8, CD20, CD68, and caspase-3 were examined under a light microscope. The histological alterations caused by the hydatid cyst were cell degeneration, necrosis, hemorrhage, congestion, and fibrosis. The findings revealed that the CD3 T cells were the most predominant inflammatory cell in hepatic mice tissue. Also, CD4 Helper T cells, CD8 Killer T cells, CD20 memory cells, and CD68 macrophages were detected in the infected mice. Most samples of mice expressed a different level of tissue necrosis where anti-caspase-3 was examined. Additionally, several mast cells in the mice liver samples were observed. By reestablishing efficient immune responses and preventing evasion mechanisms of the parasite, these findings help improve the understanding of local immune responses to CE and maybe lead to the design of novel treatments.

Keywords | Cystic echinococcosis, BALB/c, Liver, Immunohistochemistry, Apoptosis,

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INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic infection caused by the dog tapeworm *Echinococcus granulosus* metacestodes. The World Health Organization listed it as one of the twenty neglected tropical diseases (WHO, 2010). It is a significant helminthic disease, representing worldwide public health and socioeconomic concern (Wen et al., 2019). The life cycle of *Echinococcus* involves a definitive host (usually dogs and other carnivores) and an intermediate host (such as sheep, goats, or cattle). Humans act as accidental intermediate hosts since they acquire the infection like other intermediate hosts; however, they are not involved in transmitting the parasite to the definitive host (Shnawa et al., 2022a). The liver is the organ that is most frequently impacted by hydatid cysts (50–70%), followed by the lungs (25%), spleen, kidneys, heart, bones, CNS, viscera, and other organs (Kammerer and Schantz, 1993,

Bhutani and Kajal, 2018). The *E. granulosus* hydatid cyst comprises an external non-cellular laminated layer and an internal cellular germinal layer. The cyst of CE grows gradually, and the cyst is shielded by adventitial (fibrous) layers derived from the host. The host's local tissue response to the parasite led to the development of this fibrous capsule (Díaz, 2017). Generally, the formation of a membrane attack complex and the induction of inflammatory response on the parasites' surface are the first steps in the complement system's defence against invasive parasites, which lead to parasite eradication (Shani et al., 2012). In fertile hydatid cysts, buds form in the germinal membrane and develop into brood capsules within which protoscoleces are produced. Compared to incidental (humans) or aberrant (experimental animals) hosts, the parasite causes only mild to acute pathological changes in its natural hosts, including herbivorous in domestic and sylvatic habitats (Thompson, 2013). Mnati et al. (2020) concluded that the hydatid cyst leads to several histopathological effects in the hepatic tissues of cattle and sheep. The results include inflammatory cells infiltration, necrosis, hepatic tissue degeneration, fibrosis, portal vein congestion, and dilation of the bile duct. The hydatid cysts can rupture, resulting in spillage of hydatid fluid with protoscoleces, which produce new infections and consequently cause anaphylactic reactions. Cysts can also cause irreversible damage to the organs (Beigh et al., 2018a).

The parasite creates lesions in the form of cysts filling with hydatid fluid in the liver, which causes tissue damage. An outer layer of the host-formed local inflammatory cells surrounds these lesions, where lymphocytes, polymorphonuclear, eosinophils, and macrophages can be found (Atmaca, 2022). The layers of the hydatid cyst and the hydatid fluid contain a variety of antigens that are highly immunogenic in *Echinococcus* and cause inflammatory cellular reactions, the production of particular antibodies, and T-cell and other cellular immune mechanisms in human. Still, the parasite can survive in mammalian hosts for many years, suggesting that the immune system's antiparasitic response can be modified (Shnawa et al., 2021) *E. granulosus* may use either immunomodulation, in which the parasite actively engages the host immune system to lessen the effects of an immune response, or passive escape, in which the parasite transforms into a hydatid cyst and escapes the harmful effects of an immune response (Zhang et al., 2003; Siracusano et al., 2012; Jalil et al., 2022). There are differences in the interaction between the intermediate hosts and hydatid cysts, and the reasons of these variations are not entirely recognized. Nevertheless, *Echinococcus* genotype and host-associated factors are proposed to be participated. It has been revealed that in infected sheep, antibody production may be supplemented by a cellular immune response involving polymorphonuclear, lymphocytes, eosinophils, along with macrophages (Rogan et al., 2015). Also, this has been documented previously in protozoal parasitic diseases within humans and animals (Shnawa, 1995, Shani et al., 2012).

This study sought to assess the local immune cell responses in experimental murine cystic echinococcosis by categorizing immune cell subtypes using immunohistochemistry and detecting tissue degeneration and necrosis caused by hydatid cysts in the liver of infected mice.

MATERIALS AND METHODS

Experimental animals

Healthy BALB/c mice (females and males, 6-week-old) were purchased from Hawler Medical University. The mice were bred and maintained in the animal house at Biology Department, Science Faculty, Soran University-Erbil, Iraq. The mice were kept in climate-controlled housing at 20–25 °C with a 12-hour cycle of darkness and light. The mice were also given access to clean water and a standard diet. All animals were handled following the regulations of the ethics committee (issued by the scientific research committee: Faculty of Science at Soran University: 1/ 1/178 on October 2021).

Infection of BALB/c Mice with Secondary Hydatid Cysts

Sheep-infected livers with hydatid cysts were collected from two slaughterhouses in Soran City-Erbil and Erbil province. The protoscoleces were aseptically isolated, and a 0.1% eosin stain was used to assess their viability. While viable protoscolices are colourless, dead protoscolices absorb eosin and turn red (Jalil et al., 2021).

Twenty healthy BALB/c mice, weighing 25 ± 5 gm and six weeks old, were used for the secondary hydatid cyst infection. Each mouse received an intraperitoneal injection containing 1500 protoscoleces aseptically collected from naturally infected sheep livers. The injected mice were followed for six months to develop hydatid cysts (Hamad et al., 2022).

Hydatid cysts collection and histopathological analysis

Infected livers of sheep with hydatid cysts were collected from two slaughterhouses in Soran City-Erbil and Erbil province and immediately delivered to the laboratory. Mice samples are collected after six months of infection in the animal house of the Faculty of Science, Soran University. All infected livers were isolated, and small portions were fixed with 10% neutral buffered formalin for 24 hours. Following this, paraffin-embedded blocks are prepared and stored in a dry and cool environment until the examination.

After processing, five µm sections were sectioned using a microtome, and histopathological analysis was performed using standard hematoxylin and eosin (H&E) staining. The stained sections were evaluated and examined using a light microscope under different magnification powers. Also, toluidine blue staining was performed to detect mast cells (Puebla-Osorio et al., 2017).

Immunohistochemical (IHC) analysis

The paraffin blocks were cut in 3 µm sections using a Semi-automated microtome (Thermo Fisher Scientific, USA), deparaffinized at a 68 °C oven and hydrated through series of alcohol gradients. Immunohistochemical staining was done manually with an Ultra Vision Detection System kit (Thermo Fisher Scientific, USA). The reagents in this kit constitute a labelled streptavidin-biotin immunoenzymatic antigen detection system. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen. This biotinylated secondary antibody reacts with the primary antibody, enzyme-labelled streptavidin, and substrate-Chromogen. The liver samples were examined for CD3 T cells (Polyclonal Rabbit anti-human CD3, DAKO, Denmark), CD4 helper T cells (Monoclonal Mouse Anti-Human CD4, DAKO, Denmark) and CD8 killer T cells (Thermo Fisher Scientific, USA) to determine T cells. Also, the samples were examined for determining the B cells by CD20 memory cells (Monoclonal Mouse Anti-Human CD20, DAKO, Denmark). The macrophages were determined by CD68 (Mouse Monoclonal anti-CD68, Genmed, Germany). Moreover, all the samples were examined with caspase-3 to determine tissue degeneration and necrosis using (Rabbit Anti-Caspase-3 Monoclonal Antibody, SUN LONG, China).

Semi-quantitative microscopic grading and scoring

According to epithelial cell degeneration, necrosis, vascular congestion, hemorrhage, and inflammatory cell infiltration, hepatocellular was graded. The mentioned parameters were assessed and counted as mild, moderate, and severe, as follows: (–) normal histology; (I) mild <25%; (II) moderate 25– 50%; (III) severe >50% of the tissues affected (Hassanen et al., 2019). The grading and scoring were performed under 40x magnification for the inflammatory cells with stained cytoplasm, and about ten microscopic fields were randomly selected in each round (Vatankhah et al., 2015). The expression of CD3, CD4, CD8, CD20, and CD 68 were examined by immunohistochemistry using a Mouse Monoclonal antibody. According to Dako's recommendation, a method was performed to stain tissue with Anti- CD3, CD4, CD8, CD20, and CD68 antibodies. The sections of the positive immunohistochemical scoring system were implemented. Negative control was performed in parallel. Immunohistochemistry analysis for all CD showed dark-brown staining of specific structures became apparent (cytoplasmic reaction for caspase-3 and nucleus staining for CD3, CD4, CD8, CD20 and CD68), and then they were considered positive. Then, the number of immunopositive cells in ten microscopic fields under the 40× lens was estimated. The percentage of immunopositive cells in ten microscopic fields at 400 × magnification was calculated: absence «–», weak «+» (1–10% of cells), moderate «++» (11–50% of cells), and an intensive «+++» (≥51% cell) reactions (Demyashkin et al., 2020). Additionally, mast cells were scored by several fields (minimum five fields) of 40X magnification evaluation, and a mean was determined for each (Patel et al., 2012). Immunohistochemical cytoplasmic staining for all caspases-3 was scored by visual assessment of ten 40X microscopic fields by counting brown stained cytoplasm as follows: (score 1) 0-30%, (score 2) 31-70% and (score 3) >71% of immunolabelled cells (Ummanni et al., 2010). absence «–", – weak «+» (1–10% of cells), moderate «++» (11–50% of cells), an intensive «+++» (≥51% cell) reaction.

RESULTS

Secondary hydatid cysts infection of BALB/c mice

According to the findings of this research, depicted in Figure 1, A, after six months, an experimental mouse infection with cystic echinococcosis was confirmed following intraperitoneal injection of BALB/c with live protoscoleces taken from the liver of sheep infected with *E. granulosus* hydatid cysts, as shown in Figure 1, B.

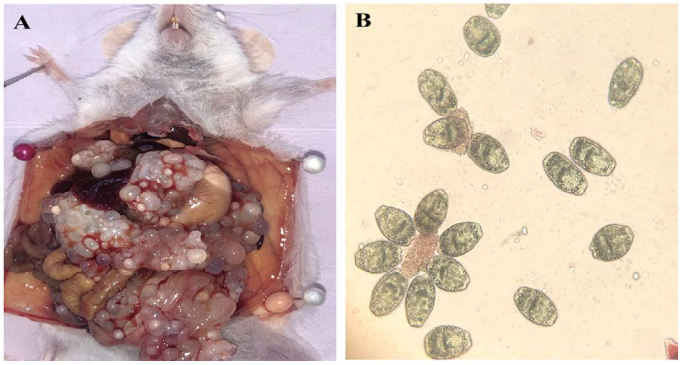

Figure 1: (A) Experimentally heavily infected mouse with hydatid cysts. (B) Protoscoleces were collected from *E.granulosus* infected sheep liver.

Histopathological alterations

The liver section of the uninfected mice showed normal hepatic tissue, as depicted in Figure 2, A. In contrast, The histological section of hepatic tissue of infected mice displayed secondary hydatid cysts with distinct germinal and laminated layers, as shown in Figure 2, B. Moreover, Fig-

ures 3, A and B illustrate different sizes of secondary hydatid cysts with their characteristic feature.

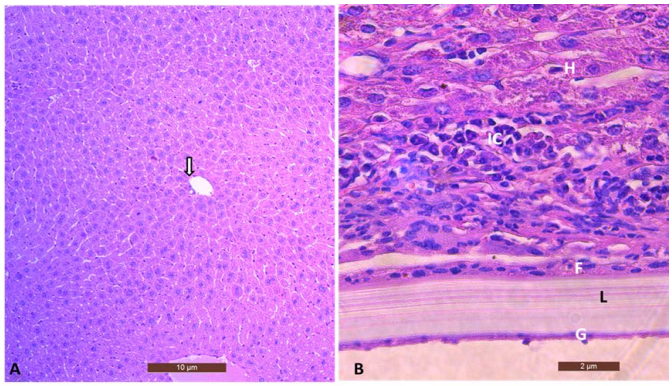

Figure 2: (A) Sections from the liver of the control uninfected mouse showed central vein (arrow) and hepatic cells(10X), Scale bar= 10µm. (B) Section of mice infected liver with hydatid cyst showing characteristics cyst layers laminated layer (L) and germinal layer (G) surrounded by fibrous tissue (F) with intense inflammatory cell infiltration (IC). (40X), Stained with H&E. Scale bar = 2µm.

Several histopathological changes were detected in the hepatic tissue of infected mice, including infiltration of inflammatory cells surrounding the hydatid cyst with degenerated hepatic tissue. Also, the liver showed an accumulation of giant and tissue necrosis adjacent to the hydatid cyst, as illustrated in Figure 4. Additionally, Figure 5 depicts the infiltration of many inflammatory cells. Moreover, more histopathological alterations were observed in the infected liver with hydatid cyst, including vacuolated hepatic cells expressing hydrophobic change, necrosis, hemorrhage, initiation of fibrosis, and vascular congestion, as shown in Figure 6.

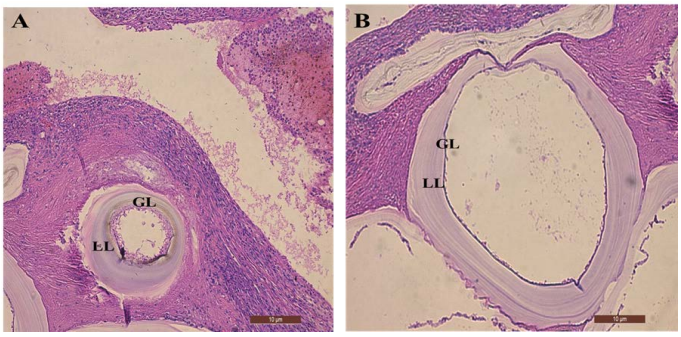

Figure 3: (A) and (B) show the secondary hydatid cyst in mice liver tissue of different sizes with (GL) germinal layer and (LL) laminated layer (10X), Stained with H&E. Scale bar = 10 µm

Immunohistochemistry and immunohistochemical scoring

Six biomarkers were examined, which are CD3, CD4, CD8, CD20, CD68 and Caspase-3. CD3 was the most predominant T lymphocyte in mice. In mice, the most

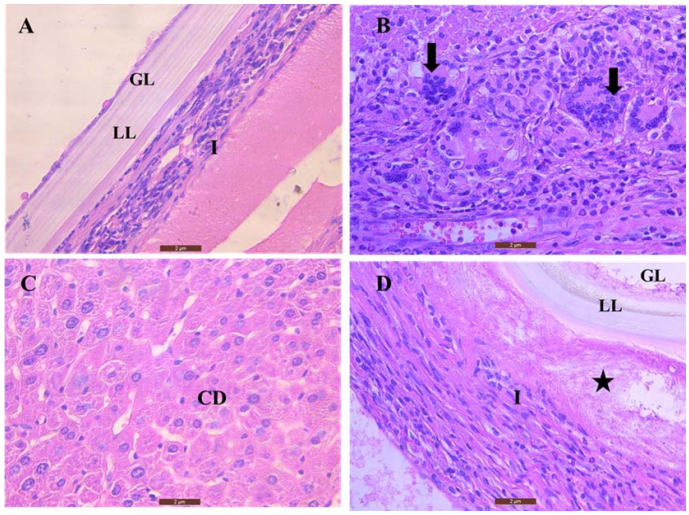

Figure 4: Sections of mice liver stained by hematoxylin and eosin (A and D) show the hydatid cyst layers: (GL) is the germinal layer, (LL) the laminated layer, and (I) infiltration of inflammatory cells that surround the hydatid cyst, the star indicates dead degenerated hepatic tissue (B) Shows accumulation of giant cells (arrows). (C) Illustrates cell degeneration (CD) and tissue necrosis adjacent to hydatid cyst (40X). Scale bar= 2µm

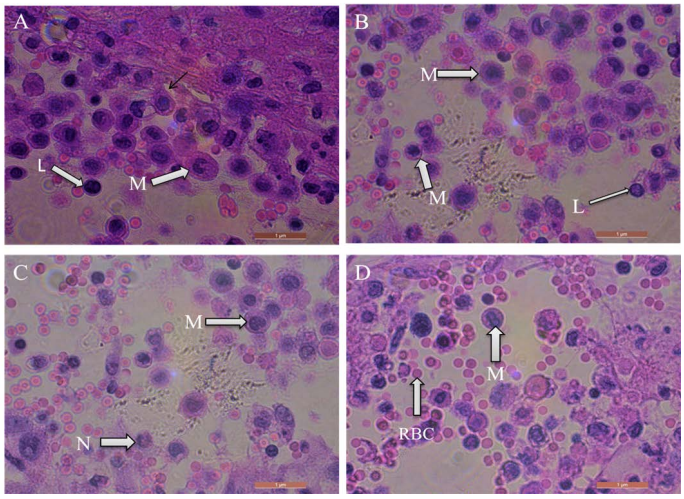

Figure 5: Sections of mice livers infected with hydatid cysts show infiltration of different inflammatory cells like macrophages (M), neutrophils (N), lymphocytes (L), and Red blood cells (RBC), Stained with H&E. scale bar = 1 µm

predominant inflammatory cell was CD3 mean of 32.72±10.04, followed by CD68 inflammatory cells, with a mean of 28.54±9.96, as illustrated in Table 1 and Figures 7 and 8. In addition, CD4 was a mean of 28.33±11.62 and CD20 with mean of 25.48±12.18, and CD8 had a mean of 28.33±11.62. All inflammatory markers were recorded as moderate (11–50% of cells), as shown in Figures 8 and 9. Expression of Caspase-3 in 70% of the samples was scored 2 (from 31% to 70%), and 30% scored 1 (from 0% to 30%). The number of mast cells per field was 7.6±1.14, which indicated a high number of mast cells in mice tissue in

response to hydatid cyst infection, as shown in Figure 10. Expression of Caspase-3 in 60% of the liver samples was scored 2 (from 31% to 70%), and 40% scored 1 (from 0% to 30%). The number of mast cells per field was 8.5±0.07, which indicated a high number of mast cells in mice tissue in response to hydatid cyst infection, as shown in Table 2 and Figure 10.

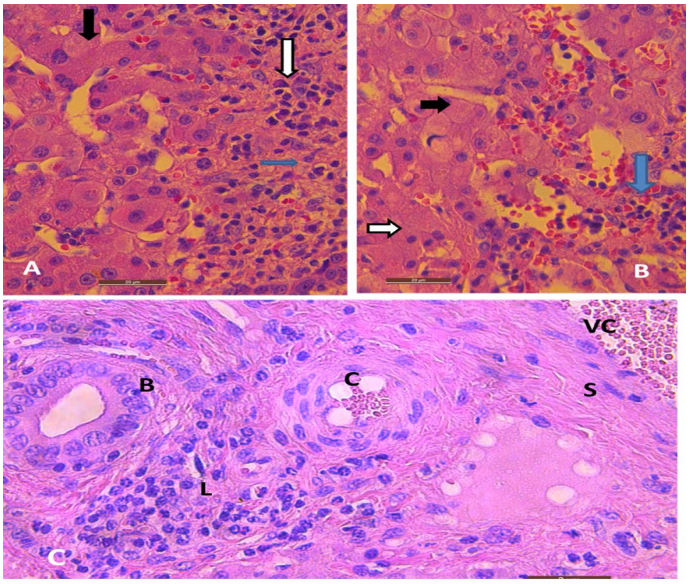

Figure 6: Liver of mice infected with hydatid cysts stained by hematoxylin-eosin. (A) Section depicts vacuolated hepatic cells expressing hydrophobic change (black arrow), necrosis (blue arrow), and Chronic infiltration of inflammatory cells (lymphocytes) (white arrow). (B) It shows vacuolation as reversible injury in hepatocytes (black arrow), necrosis (white arrow) and hemorrhage (blue arrow). (C) Illustrates bile duct (B), infiltration of lymphocytes(L), spindle shape fibroblasts (S), and vascular congestion (VC). A and B. Scale bar= 20µm, C. Scale bar= 2µm.

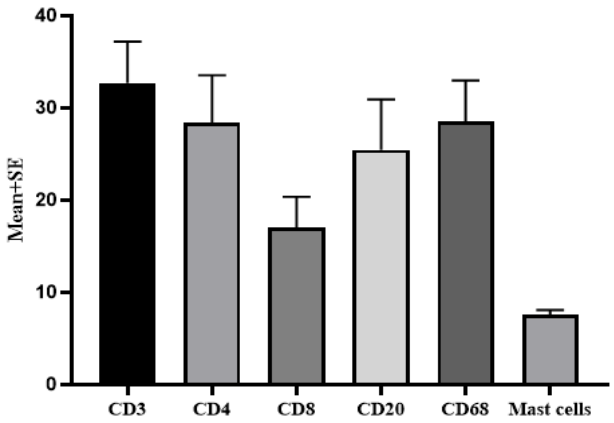

Figure 7: The Mice's liver's density of inflammatory cells and mast cells in Cystic Echinococcosis.

Table 1: Density of inflammatory and mast cells of the mice's liver in cystic echinococcosis. **Positive cells / 100 cell (mean% ±SD)**

Marker	Result
CD3	32.72±10.04
CD4	28.33±11.62
CD8	17.08±7.39
CD20	25.48±12.18
CD68	28.54±9.96
Mast cells	7.6±1.14

Table 2. Expression of caspase-3 in Mice's liver with cystic echinococcosis. Score1(0-30%), score 2 (31-70%), score 3 (71-100%).

Sample	Caspase-3 score
1	2
2	1
3	2
4	2
5	2
Control	1
Control	1

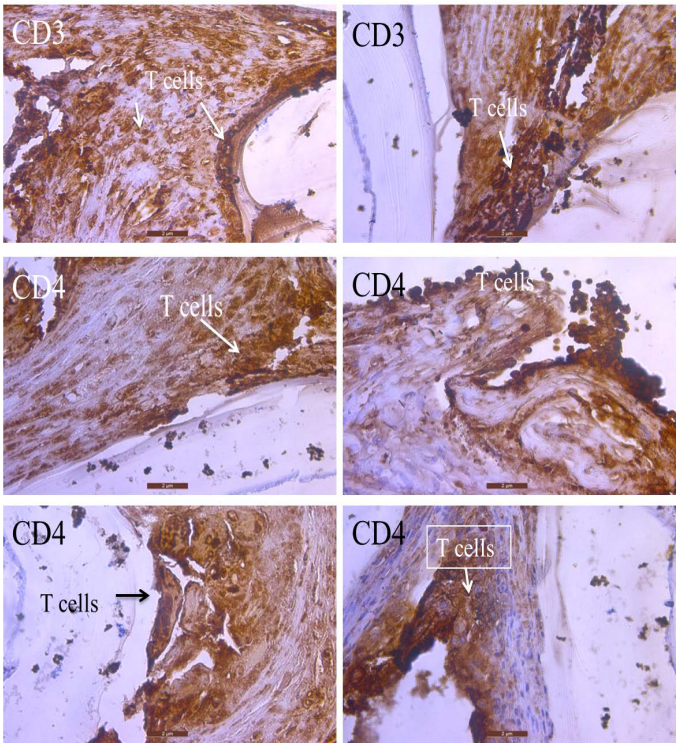

Figure 8: CD3. Localization of T lymphocytes by CD3 immunolabeled in the liver of infected mice. (40X). CD4. Show localization of T helper cells by CD4 immunolabeled in the liver of mice infected with hydatid cysts. (40X). Scale bar= 2µm

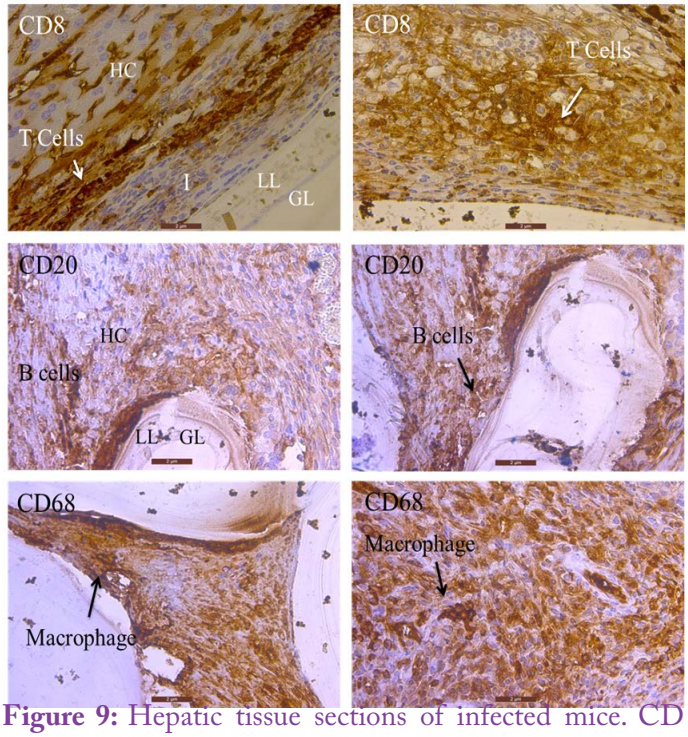

Figure 9: Hepatic tissue sections of infected mice. CD 8. Localization of killer T cell by CD8 immunolabeled. (40X). CD 20. Localization of memory cells by CD20 immunolabeled. CD 68. Macrophage by CD68 immunolabeled (40X). Scale bar= 2µm

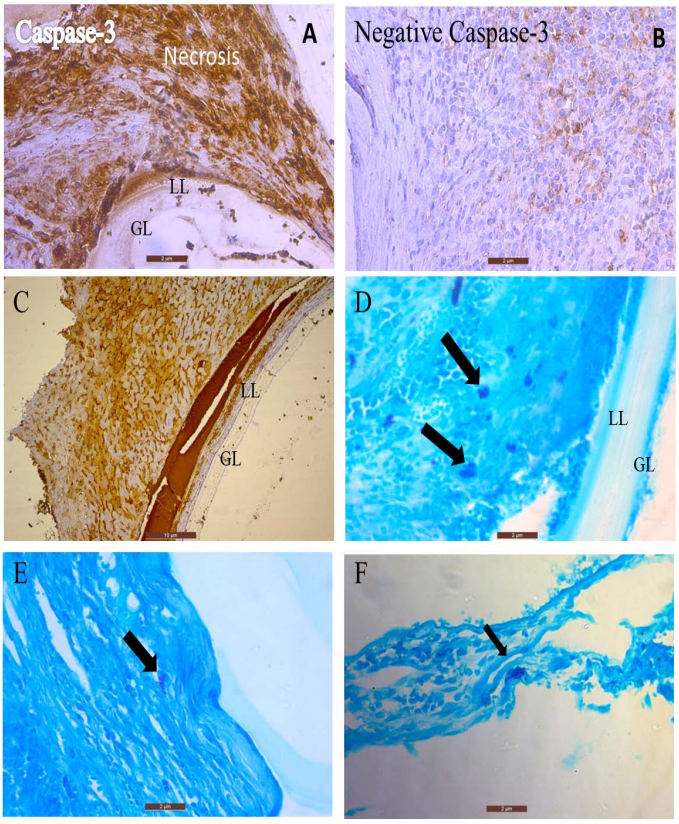

Figure 10: (A-C) Expression of caspase-3 by immunohistochemistry in mice liver with hydatid cyst (40X) **(D-F)** Section of mice liver presented mast cells stained with toluidine blue showing intracytoplasmic granules. (40X). Scale bar= 2µm

DISCUSSION

The most common organ infected is the liver, then the lung in sheep and humans. Two of the experimentally infected mice in this study displayed liver infection. In three mice, we observed infection of all organs after intraperitoneal injection with protoscoleces of *E.granulosus*, as shown in (Figures 1, A). The results revealed that the cyst has a germinal layer and a laminated layer, as determined by the hematoxylin-eosin. Protoscolices and hydatid fluid are produced by the germinal layer and contain antigens that alter the liver tissue's histology. The present histopathological study of the liver samples of mice showed cell degradation (necrosis), fibrosis, congestion, and infiltration of inflammatory and mast cells observed in different degrees around the fibrous capsule. This result agreed with the findings of (Beigh et al., 2018a; Hansh, 2019; Mnati et al., 2020) (Hansh, 2019). Mast cells were observed using toluidine blue as a specific stain, and our result indicated that the number of mast cells was high in mice liver tissue. This result agreed with (Beigh et al., 2018b), who documented the mast cells in sheep liver tissue. According to our data, the inflammatory cells reacted clearly in experimentally infected mice. This fact has also been demonstrated previously, as the experimental infection expressed more inflammation than the naturally infected sheep had (Vatankhah et al., 2015).

In the current experimentally infected mice, CD4, CD8, CD20, and CD68 showed positive reactions, and CD4 was the second most reactive biomarker that expresses the activation of T lymphocytes as a response to the hydatid cyst. In the murine model, the CD4 and CD8 scored remarkably varying degrees of susceptibility to disease, and the parasite-induced a less severe immune response. The immunohistochemical study for hydatid cysts of mice samples showed that CD3 was the most predominant cell infiltrated in the tissue surrounding the hydatid cyst. The same result was recorded in several previous studies (Ibrahim and Gameel, 2014; Vismarra et al., 2015;Al Malki and Ahmed, 2022). Hou et al. demonstrated that T cells, specifically CD4+ T cells, were crucial in developing the immune environment in the liver in CE patients and *E. granulosus*-infected mice (Hou et al., 2022). Also, the CD20 was positive in the mice samples of our study, indicating a humoral immune response to the hydatid cyst. While in sheep, CD20 showed a negative reaction, as mentioned previously by (Ibrahim and Gameel, 2014), indicating the absence of B lymphocytes. Also, Jiménez et al. reported that CD3 T cell is predominant in sheep compared to the CD79 B lymphocytes (Jiménez et al., 2020) to 10 weeks post-infection, which is consistent with our findings (Breijo et al., 2008).

According to the current findings, the liver tissue has undergone numerous changes, including fibrosis, necrosis, degeneration of the liver tissue, fibrosis around the portal veins, congestion of the portal veins, an increase in the number of bile ducts, and misalignment of the hepatic cords. In this regard, Mnati et al. (2020) mentioned that the enlarged cysts caused pressure that led to the hepatic lines becoming misaligned, pressed on the hepatocytes, and congested the portal veins. In another investigation, liver sections taken from the regions close to the cyst wall revealed fibrosis, cellular infiltration of prominent macrophages, lymphocytes, and plasma cells, congestion, hemorrhage, hepatocyte necrosis, and atrophy. These changes were most visible on the inner side of the fibrous capsule. There was dilatation of sinusoids and central veins, and fibrosis was also seen in the portal area (Beigh et al., 2017).

The results of the current study revealed that the hydatid cysts in the infected mice were sterile. Numerous investigations have demonstrated that apoptosis-inducing factors are expressed at higher levels in the germinal layer of sterile cysts than in fertile cysts, and anti-apoptotic materials are expressed at higher rates in fertile cysts, which may have an impact on hydatid cyst infertility (Moghaddam et al., 2019). Inflammatory CD3+ T cells and CD19+ B cells were the two most common inflammatory cells in all groups. According to (Jafari et al., 2019), various immune cells are involved in the local response to human echinococcosis. According to immunology, hydatid cysts release and make a variety of immune-modulatory molecules available to the host's immune system. Siracusano et al. (2008) found that these molecules target both humoral and cellular reactions and activate innate and adaptive immune responses. For a deeper understanding of the interaction between the host and the parasite as well as the variables that affect this interaction, research into the pathologic pathways of cell death is also crucial. In particular, in the liver and lungs, factors that affect the enzyme activity in hydatid cysts can cause cystic infertility and stop the spread of the disease (Moghaddam et al., 2019).

Immunohistochemical techniques utilizing anti-INOS besides anti-IL-10 antibodies were used to identify the different kinds of macrophages participating in the local immune mechanisms against hydatid cysts in sheep's hepatic tissue. They demonstrated that the local immune response to hydatid cysts coexists with the activation of Th1 and Th2 immune responses (Atmaca, 2022).

Our data showed a high percentage of caspase-3 in mouse liver samples, demonstrating the hydatid fluid antigens' capacity to induce apoptosis in surrounding tissue. Hussein et al. (2020) mention it is a parasite strategy for surviving and controlling the immune response. The same authors concluded that, following the induction of a cystic echinococcosis mouse model, The parasite could stimulate apoptosis in hepatic and spleen tissues (Hussein et al., 2020). Additionally, immunohistochemistry analysis revealed that cytokeratin and caspase3 levels were higher in echinococcus tissues than in normal tissues. Hepatic echinococcosis increases the expression of cytokeratin and apoptosis-associated molecules, which indicate liver damage (Yang et al., 2022). Moreover, (Hu et al., 2011) mentioned some drug-induced apoptosis of E. granulosus protoscoleces and proved the presence of a CED-3-like apoptosis gene in the protoscoleces. Therefore, apoptosis is a possible mechanism for the action of some drugs, including the ZnO-nanoparticles tested against *E.granulosus* (Shnawa et al., 2022b).

It has long been believed that the adventitial layer of CE cysts serves as the parasite's protective, fibrous capsule derived from its host. Although many fertile CE cysts still display this feature, the adventitial layer is currently assumed to result from a local immune response that includes granulomatous tissue, lymphocytes, eosinophils, plasma cells, and other innate immune cells that target both fertile and sterile CE hydatid cysts. In contrast to fertile cysts, the density of inflammatory cells was significantly higher in the small and sterile cysts, particularly lymphocytes and multinucleated giant cells (Barnes et al., 2011; Hidalgo et al., 2019; Hidalgo et al., 2021). These facts also support our findings regarding the inflammatory infiltrates involving mainly macrophages and T cells.

CONCLUSION

Among the intermediate hosts, humans are harmed when hydatid cysts develop, predominantly in the hepatic and pulmonary tissues. Many known pathways enable the cysts to survive in different hosts while the host immune response is suppressed. Immune response modulation and controlled cell death fundamentally influence cyst development, growth, and pathogenesis. This work proved that the histological alterations caused by the hydatid cyst were degeneration, necrosis, hemorrhage, congestion, and fibrosis. The findings revealed that the CD3 T cell was the most predominant inflammatory cell in hepatic mice tissue. Also, CD4 Helper T cells, CD8 Killer T cells, CD20 memory cells, and CD68 macrophages were detected in the infected mice. Most samples of mice expressed a different level of tissue necrosis where anti-caspase-3 was examined. Additionally, several mast cells in the mice liver samples were observed. By possessing efficient immune responses and preventing evasion mechanisms of the parasite, these findings may improve the understanding of local immune responses to CE and maybe lead to the design of novel medications.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

NOVELTY STATEMENT

The current work's novelty is the immunohistochemical characterization of immune response and apoptosis expression in an experimental model of hepatic cystic echinococcosis.

AUTHORS CONTRIBUTION

Shnawa BH and Alrawi RA suggested and designed the study, and Hamad BSh performed the practical parts. All authors interpreted the data and wrote as well as revised the manuscript.

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