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Apoptotic Gene Expression in Sheep Hepatocytes during *Fasciola hepatica* Infection (Fascioliasis)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The main objective of this work was to investigate the apoptotic genes of sheep liver hepatocytes to elucidate the apoptosis pathway mechanisms during *Fasciola hepatica* infection using molecular and serological techniques.
Study Design: This is a laboratory based study whereby *F. hepatica* infected liver specimens were used.
Methodology: Total RNA was extracted from fresh-frozen liver tissue. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to investigate the genes encoding the mRNA for following proteins: 18S; Bax; Bcl-2; Caspase-3. Additionally, the concentration of total protein in each sample was estimated spectrophotometrically. Polyclonal antibodies were produced by immunizing rabbits and diluted with 5% skimmed milk in PBS containing 0.1% Tween 20 (1:1000). The detection of the reacted antigen/antibody products was performed using immunoblotting technique were used to assess the quantification of protein kinetic to apoptotic genes. Data obtained from this investigation were analyzed using Portable IBM SPSS 2006 Statistics software.
Results: We investigated the apoptotic gene expression in sheep liver hypatocytes during

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F. hepatica infection by mRNA expression of three genes involved in apoptosis; Bax, Bcl-2 and Caspase-3 and histopathological parameter after infection with *F. hepatica* in the liver cells. Quantitative real-time PCR, histological examination and immunoblotting were used to quantify apoptotic genes, histopathology and protein kinetic, respectively. *F. hepatica* infection induces apoptosis in the liver cells via Bax, Bcl-2 and Caspase-3 genes.

Conclusions: *F. hepatica* infection induces apoptosis in the liver cells via Bax, Bcl-2 and Caspase-3 genes and it precedes necrosis. Thus, this study suggests that the induced apoptotic gene expression was due to the outcome of *F. hepatica*.

Keywords: Fasciola hepatica; fascioliasis; hepatocytes; apoptotic genes; PCR; liver; sheep.

1. INTRODUCTION

Fascioliasis is a disease of ruminants caused by two major parasitic trematodes, *Fasciola hepatica* and *F. gigantic* [1]. In spite of decades of intense research, fascioliasis is one of the most common helminth infections of sheep and cattle, and can result in productivity losses impacting on the economy of the livestock industry. In addition, fasciolosis is also considered a dangerous public health problem in humans [2], with an approximately 17 million people considered to be infected [3] and more than 180 million at risk of infection worldwide. Humans may get infection through the consumption of raw vegetables or by drinking water contaminated with fasciola metacercariae. Moreover, human fascioliasis caused by *F. hepatica* has been recently recognized as an emerging/re-emerging zoonotic disease in many countries [4]. Infections are hyper-endemic in areas of South America, Iran, Egypt, Portugal and France [5]. Human fasciolosis has also been noticed in the European countries of France and Spain [6]. Economic losses include costs of anti-helmmthics and land drainage, and losses in productivity as a result of mortality, including the reduction in meat, milk and wool production [7]. It has been estimated that losses due to fascioliasis might amount to more than $200 million dollars annually [8]. Fascioliasis is widespread in Ireland and is a particular problem in areas where high rainfall and poor draining soils combine to exacerbate the situation. However, the prevalence of infection is significantly higher in developing countries [9]. Although many mammalian species can be infected with *F. hepatica*, there is variation in the level of susceptibility to infection, and in the ability to mount an effective immune response for example, sheep often die from acute fasciolosis, while some infections may last for 11 years [10]. *F. hepatica* often causes chronic infection, which indicate the development of strategies by the parasite to change/inhibit the host immuneresponse [11,12]. Furthermore, liver flukes possess an ability to disable in vitro immune effector cells, for example by inactivating the toxic reactive oxygen products of the respiratory burst of leukocytes (eosmophila and neutrophils) and macrophages or reactive nitrogen intermediates generated by macrophages [13]. Oxygen scavenging enzymes such as superoxide dismutase (SOD) may be involved in the inactivation of oxygen species [14,15] and Piedrafita [15] observed *F. hepatica* peroxiredoxin may play a role in the flukes defense against ROS, generated as a by-product of intracellular metabolism, from the metabolism of certain pharmacological agents and from the effector arm of the host immune system [16].

Apoptosis, programmed cell death, is an active form of cell death which is linked intimately with both physiology as well as pathology in variety of cellular systems [17,18]. The dysregulation of liver apoptosis during fascioliasis is a critical event in liver pathology. Apoptosis does not only play an important role in development and tissue homeostasis but is also involved in a wide range of pathological conditions. Reactive oxygen species (ROS) have been involved in the apoptosis induced by different stimuli as well as the pathologic cell death that occurs in many diseases [19]. Apoptosis may result via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. The death receptor pathway comprises Fas (CD95/ Apo-1) and TRAIL (Apo-2) and this pathway is activated when ligands specific for either Fas or TRAIL bind to their respective receptors, leading to activation of Caspase-8, which eventually activates Caspace-3 [20]. Furthermore, mitochondrial pathway of apoptosis is working by the down-regulation of anti-apoptotic proteins (such as, Bcl-2, Bcl-xl) and/or up-regulation of pro-apoptotic proteins (such as, Bax, Bad, Bid), which leading to the opening of mitochondrial

permeability transition pores and release of apoptosis inducing proteins (cytochrome, apoptosis inducing factor, etc.) from mitochondria [21-23]. It was also recorded that the Excretory-secretory products (ESP) from *F. hepatica* induced in vitro an early Eosinophil (Eo) apoptosis through a pathway that involved TyrK and caspase cascade activation [24]. Also, *F. hepatica* was found to be able to induce apoptosis of another essential innate immune cell (Macrophage) which play key roles in the immune response developed during helminth infections [25].

The purpose of the present study was undertaken to investigate molecular analysis involved in apoptotic genes of sheep liver hypatocytes to elucidate the apoptosis pathway mechanisms during *F. hepatica* infection.

2. MATERIALS AND METHODS

2.1 Animals and Infection

Adult *Fasciola hepatica* worms, present in the common bile duct were removed from sheep livers obtained from commercial slaughter facilities. The infected liver was incised and the flukes were removed and fixed in formalin and, stained with Haematoxylin and Eosin (H&E) for histopathological examination. Some of the samples were stored at −80°C until further use for RNA extraction.

2.2 RNA-Extraction

Total RNA was extracted from 250 mg of fresh-frozen liver tissue by 5 ml TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions, and resuspended in 200 µl RNAse-free water. RNA concentrations were quantified by optical density measurements at 260 and 280 nm. Purity and integrity of RNA was checked by 1% agarose gel electrophoresis. The extraction process was carried out in a sterile environment inside a hood, the tools were used in the extraction (pipette tips and tubes) pre-sterilized in the autoclave device. The protein precipitation step is the most important and should be accurately conducted) to ensure the purity of the RNA from protein and DNA.

2.3 Quantitative Real-time PCR

Purified RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 h and then converted into cDNA following the manufacturer's protocol using the Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism®7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Mastermix from Qiagen (Hilden, Germany). We investigated the genes encoding the mRNA for following proteins: 18S; Bax; Bcl-2; Caspase-3. All primers used for qRT-PCR were commercially obtained from Invetrogen. cDNA fragments of the 3 genes were amplified by polymerase chain reaction (PCR), using primers F(5′-CCCACCAATATAAAAGCTCCTGA-3′) and R(5′-CCAGCCCTTTCTTTACACAGT-3′) for caspace – 3 gene, F(5′- TTTGCCAGCATGTTTTCCGA-3′) and R(5′- CCAATGTCCAGCCCATGATG-3′) forbax gene. F(5′- ACAGAATCAGATAT GGAAACCCC-3′) and R(5′- CCTCAGGGCTT GCTTCACC-3′) for Bcl-2 gene, F(5′-TCATCCCTGCTTCTACTGGC-3′) and R(5′-GGGTGGGATACTTGGCAGG-3′) for GAPDH gene (housekeeping gene). Reaction specificity was checked by performing dissociation curves after PCR.

2.4 Protein Extraction

10 mg tissue samples from the left and right hepatic lobes and HLN were snap-frozen in optimal cutting temperature (Miles, Elkhart, IN, USA) then homogenized in a cold homogenizer tube containing 2 ml of homogenization buffer. Homogenates were spun at 300 rpm for 10 min. The supernatants were removed and stored at -80°C. The concentration of total protein in each sample was estimated spectrophotometrically (Gene Quant pro, Amersham, USA) at 595 nm. Equal volumes of 2x sample buffer and protein (30 µg/µl) were mixed in an Eppendorf tube and heated to 95°C for 5 min before loading [26,27].

2.5 SDS–PAGE and Immunoblotting

2.5.1 Antibodies

2.5.1.1 Primary antibodies anti-(Bax; Bcl-2; Caspase-3 and GAPDH)

Primary antibodies were obtained from Cell Signalling, USA and used to detect the Bax; Bcl-2 and Caspase-3 proteins and GAPDH protein as a reference.

Polyclonal antibodies were produced by immunizing rabbits and diluted with 5% skimmed milk in PBS containing 0.1% Tween 20 (1:1000).

2.5.1.2 Secondary antibodies (anti-rabbit IgG) HRP-linked antibodies

Secondary antibodies were obtained from Cell Signalling, USA. Antibodies were labeled with peroxidase and assayed using enhanced chemiluminescence (ECL) western blotting detection reagents obtained from RPN2106PC, USA. The mix of proteins and 2x sample buffer were separated on 30% polyacrylamide gel using a Power Pac Basic system (S.N 37S/7159, Italy) at 50 V for 1 h and then at 100 V near the end of the electrophoresis. Proteins were then blotted on nitrocellulose membrane. The nitrocellulose membrane was washed several times with phosphate buffered saline (PBS) and then incubated in 5% skimmed milk in PBS containing 0.1% Tween 20 to blocking of nonspecific sites. Membranes were incubated with primary antibodies overnight at 4°C, and then with secondary antibodies for 3 h. Membranes were then washed three times (5 min each) in PBS-T, all steps under mild agitation. The detection of the reacted antigen/antibody products was performed using enhanced chemiluminescence Western Blotting Detection Reagents according to the manufacturer's instructions [19].

2.5.1.3 Immunoblotting

The extracted protein volume was determined as 50 µg in each well according to [28]. After preparing the 10% stacking gel solution, assemble the rack for gel solidification. (10% AP and TEMED solidify the solution; therefore, both gels can be prepared at the same time, if the abovementioned reagents are not added until the end). Stacking gel solution was added carefully until the level is equal to the green bar holding the glass plates, then H2O was added to the top. After 15–30 minutes, the gel become solidified. The stacking gel was overlayed with the separating gel, after removing the water. The comb was inserted and it was ensured that there were no air bubbles until the gel is solidified. The proteins were separated by size, charge, or other differences in individual protein bands. The separated protein bands are then transferred to a carrier membrane (e.g. nitrocellulose, nylon or PVDF). This process is called blotting. The proteins adhere to the membrane in the same pattern as they have been separated due to interactions of charges. The proteins on this immunoblot are then accessible for antibody binding for detection.

2.6 Statistical Analysis

Data are expressed as the means ± standard error of mean (SEM). Data were first analyzed using Portable IBM SPSS Statistics software. A paired-sample t-test was then performed to compare the treated samples, and values of $P<0.05$ were considered to indicate statistically significant differences.

3. RESULTS AND DISCUSSION

3.1 Apoptotic Genes Expression in Sheep Liver Hepatocyte

The mRNA and protein levels of Bax, Bcl-2 and Caspase-3 genes of infected liver **'s** hypatocytes were estimated using RT-PCR and western blot analysis. The level of mRNA expression for Bax as a pro-apoptotic gene and Caspase-3 as an executioner gene were significantly ($P <0.05$) increased after infection compared with control samples (Fig. 1). The level of mRNA expression for Bcl-2 as anti-apoptotic gene was significantly ($P <0.05$) and dramatically increased after infection compared with control samples (Fig. 1). Representative Western blots of Bax; Bcl-2 and Caspase-3 proteins in liver **'s** hypatocytes are shown in (Figs. 2 and 3). The results show a decreased Bax protein in infected samples compared with control samples. Thus, our findings indicating decreased level of Bax protein in the liver during *F. hepatica* infection. The levels of Bcl-2 and Caspase-3 were increased after infection compared with control samples. The GAPDH protein was used as a control. The apoptotic activities precede necrosis. These results coincide with the findings of Escamilla et al. [29] who evaluated the number of apoptotic eosinophils in the livers of sheep experimentally infected with *F. hepatica* during the migratory and biliary stages of infection. They were the first to report apoptosis induced by *F. hepatica* in sheep and the first study reporting apoptosis in eosinophils in hepatic inflammatory infiltrates *in vivo*. Their results revealed that numerous caspase-3+ eosinophils were mainly found at the periphery of acute hepatic necrotic foci. The percentage of caspase -3+ apoptotic eosinophils in the periphery of necrotic foci was high (46.1–53.9) at 8 and 28 dpi, respectively.

3.2 Histopathology

Lesions containing parasitic remnants or fluke eggs were rarely seen. However, surface scarring of the liver, scar tracks and granulomas within organs were the most characteristic changes seen and were the most useful for the histopathological diagnosis of the disease. The presence of flukes in the bile ducts produces fibrosis of the duct walls. The walls, however, remain pliable and expanded to accommodate the parasites and calcification was never observed. A dilated intrahepatic bile ducts, one of them containing adult flukes, are clearly seen. There is marked proliferation of bile ducts and inflammatory cellular infiltration (Figs. 4 and 5).

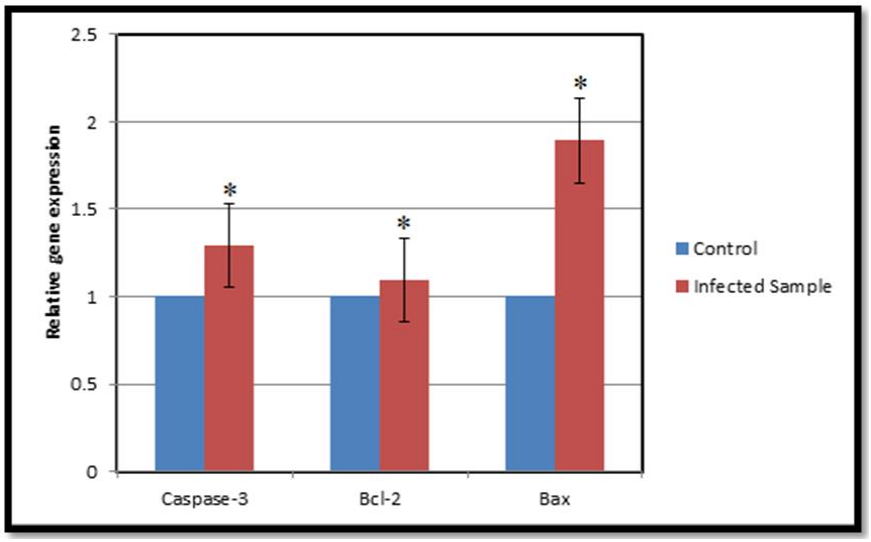

Fig. 1. RT-PCR of Caspase-3, Bcl-2 and Bax genes expression in liver of *F. hepatica* infected sheep. RNA was extracted following infection, and the relative quantity was normalized to GAPDH

*The data present are the mean ±SE (n= 30). *Significant value at (P < 0.05)*

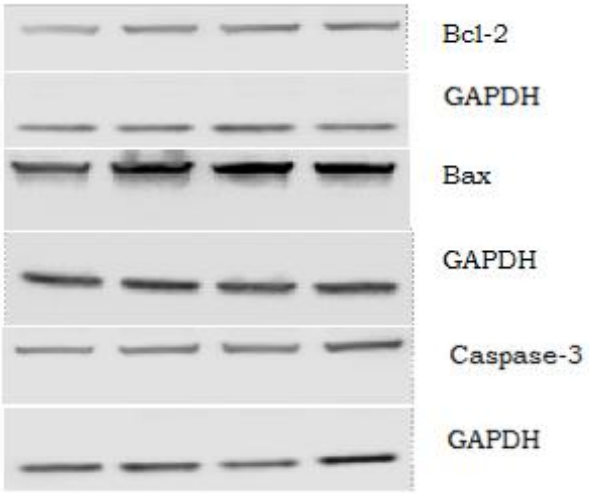

Fig. 2. Western blot analysis of Bcl-2, Bax, Caspase-3 and GAPDH obtained from cytosol of control sheep. Equal amount of protein (30 µg/lane) was electrophoresed by SDS–PAGE. Protein was transferred onto nitrocellulose membrane and probed with primary antibodies as described in the text. The immunoplot is a representative of three independent experiments with similar results

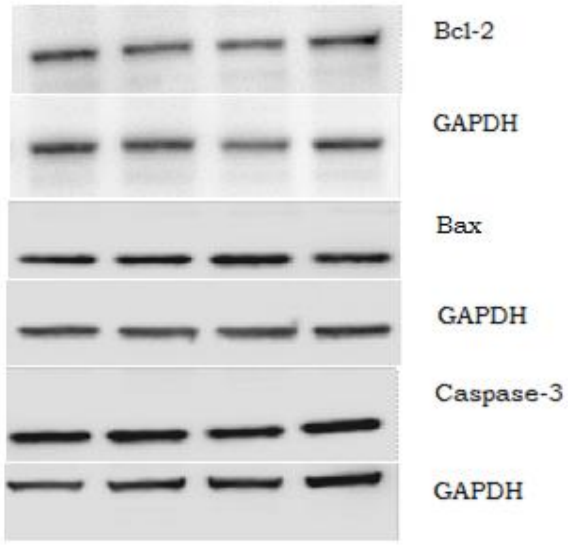

Fig. 3. Western blot analysis of Bcl-2, Bax, Caspase-3 and GAPDH obtained from cytosol of infected sheep. Equal amount of protein (30 µg/lane) was electrophoresed by SDS–PAGE. Protein was transferred onto nitrocellulose membrane and probed with primary antibodies as described in the text. The immunoblot is a representative of three independent experiments with similar results

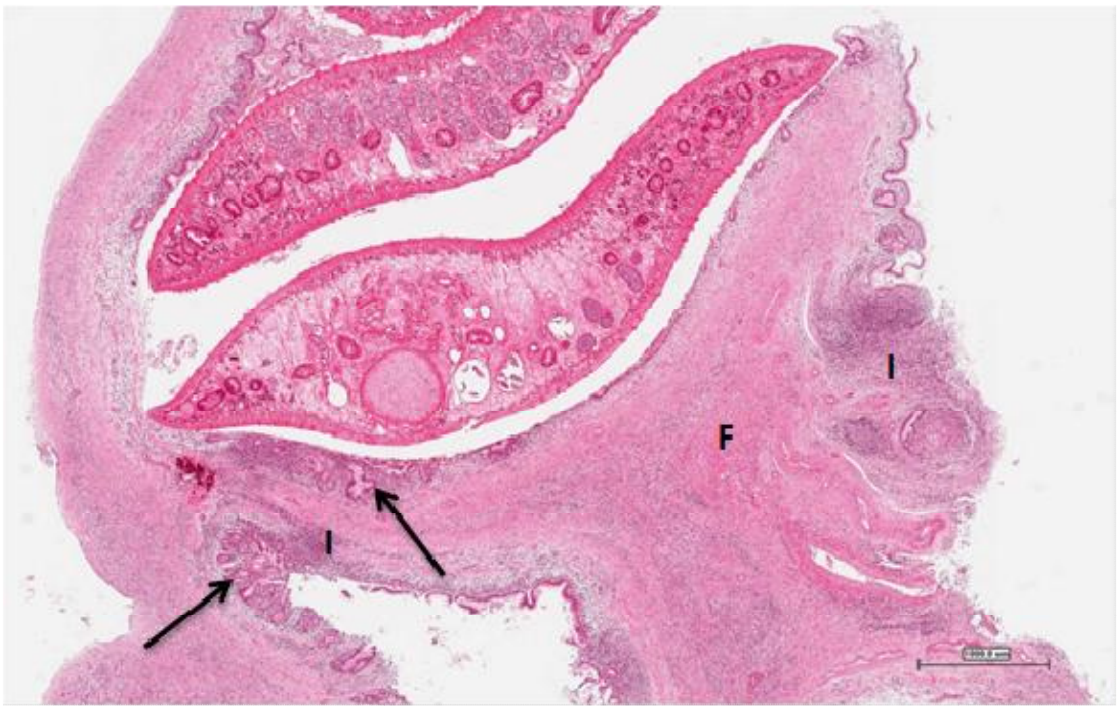

Fig. 4. A higher magnification of a section of liver showing a dilated intrahepatic bile ducts, one of them containing adult flukes, are clearly seen. There is marked proliferation of bile ducts (arrows). The dilated bile ducts are surrounded by a severe host tissue reaction comprising extensive fibrosis (F) and infiltration by inflammatory cells (I) (cholangitis). (H & E stain, scale bar = 1000 µm)

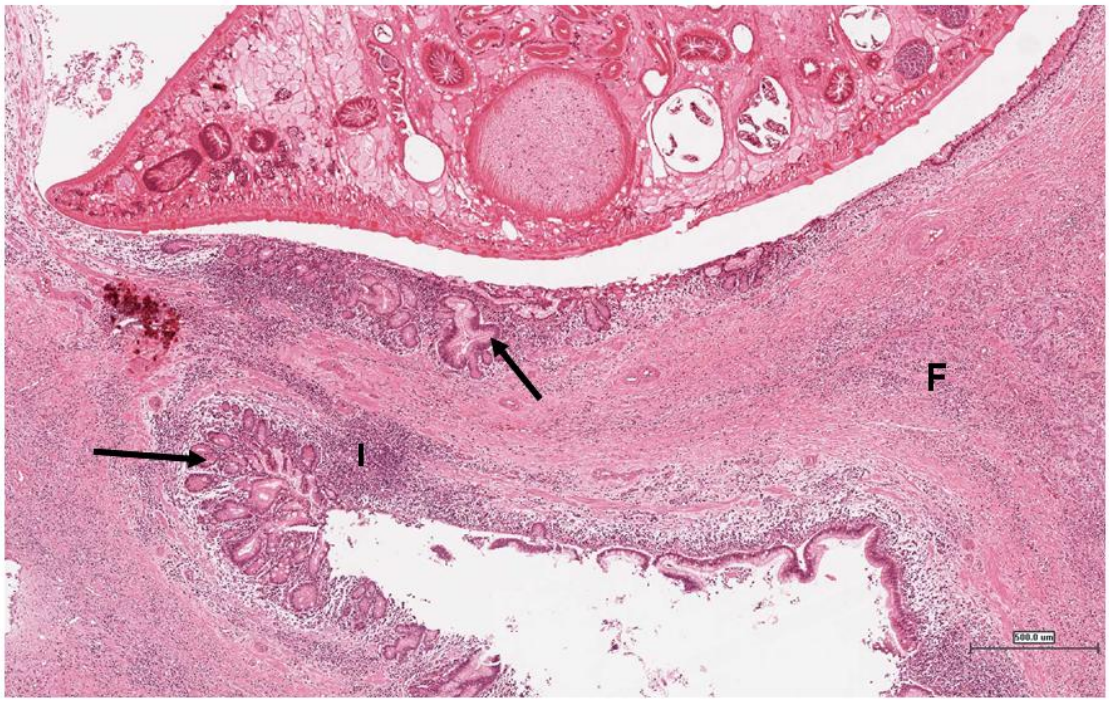

Fig. 5. Section of liver of the above photomicrograph showing clearly the marked proliferation of bile ducts (arrows), and the surrounding fibrosis (F) and inflammatory cellular infiltration (I). (H & E stain, scale bar = 500 µm)

Fasciolosis is considered one of the most widespread foodborne and waterborne trematodiasis worldwide [30]. It is caused by flukes of the genus Fasciola, being *F. hepatica* and *F. gigantica* the both species responsible for the disease, which mainly affects ruminants but can also affect humans, causing important economic losses in developing countries estimated at over 3 billion dollars per year [31,32]. In addition, *F. hepatica* is a parasitic helminth and the causative agent of fasciolosis, an emerging dangerous disease in humans [33].

Fascioliasis has been one of the main focuses of research in molecular parasitology since they are involved in key processes related to parasite invasion and survival. This study demonstrates that the infected sheep with *F. hepatica* induced apoptotic genes expression by assessing mRNA expression of three genes involved in apoptosis Bax, Bcl-2 and Caspase-3. Apoptosis is a complex cellular process whereby cells commit ‘‘suicide’’ in response to a wide variety of stimuli [34].

Although apoptosis was originally thought to be entirely distinct mechanisms of cell death, several works have shown that the processes are regulated by many of the same biochemical intermediates, including death factors and reactive oxygen species [35]. Phagocytic cells such as eosinophils and neutrophils have been shown to kill parasites by undergoing a respiratory burst and releasing such reactive oxygen species as hydrogen peroxide and superoxide anion [36-38]. A number of functions of thioredoxin have been elucidated to date, one being that of an antioxidant, as well as a modulator of apoptosis, cell growth and differentiation, and also a regulator of DNA-binding activity of several transcription factors. As shown by this study, *F. hepatica* infection significantly induces liver apoptosis mediated by oxidative stress mechanisms.

Generally, ROS generation and cytotoxins can increase in many pathological situations and cause cell death, often in a dose-dependent manner high dosages of the toxicant usually result in necrosis leading to loss of ion homeostasis, and secondly to the inability to maintain mitochondrial respiration and ATP levels essential for cellular survival [18]. The debris of necrotic tissue, eosinophil infiltration, chronic granulomatous lesions and Charcot-Leyden crystals may suggest *F. hepatica* human

[39]. However, the adult flukes may no longer survive due to starvation in the increasingly large abscess containing only necrotic-tissue debris [40]. The healing process of fascioliasis in the peritoneal nodule involves the epithelioid cells eosinophils multinucleated giant cell and fibrosis surrounding the necrotic area in the peritoneal nodule [41]. Chronic liver fluke infection raises production of the oxysterols Triol and 3K4 in the setting of chronic inflammation in the biliary system. These oxysterols induce apoptosis and DNA damage in cholangiocytes. Insufficient and impaired DNA repair of such mutated cells may enhance clonal expansion and further drive the change in cellular phenotype from normal to malignant [42]. Cell apoptosis is regulated via two major pathways: the intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. Both pathways converge at the level of active effect or caspases which cleave various cellular target proteins and then leading to apoptosis. In the liver, the apoptosis could result from a combination of both pathways: the intrinsic apoptosis pathway by generation of oxidative stress and the extrinsic apoptosis pathway by activation of Kupffer cells which can secrete TNF [18]. The mitochondrial apoptotic pathway plays a critical role in liver cell death during malaria infection [43]. Pathological consequences of the infection occur mainly in the liver, extrahepatic bile ducts and gallbladder, and have been described in both humans, experimentally infected hamsters and other animal models [44-46]. Our findings on the histopathology shown by the pathogen of *F. hepatica*, enters bile ducts and thus during feeding, blood as well as tissue debris is actively ingested by the flukes [47,48], exposing them internally to various host cellular components. Consequently, both the external surface of the fluke as well as the internal lining of its digestive tract are bathed in host cellular debris and metabolic products.

4. CONCLUSION

In conclusion, under our experimental conditions, *F. hepatica* infection induces apoptosis in the liver hepatocytes via Bax, Bcl-2 and Caspase-3, the apoptotic event precedes necrosis. In contrast, we have to bear in mind that apoptosis detection tests may reveal different results because various parasites and their host organisms possess forms of thioredoxin with similar sequence homologies. But only recently have endeavors been directed towards a better understanding of the relationships between thioredoxin and how both the hosts and the parasites infecting them utilize this protein in their protection and defense against each other some of specific targets are affected by different factors like caspases enzymes while other targets are not affected.

A novel study assessed the nature of the local immune response by examining the distribution of CD2+, CD4+, CD8+ and γδ+ T lymphocytes along with IgG+, IL-4+ and IFN-γ+ cells in the liver and hepatic lymph nodes (HLN). Results of that research revealed that immunization with rSm14 in Quil A adjuvant induced a reduction in gross hepatic lesions of 56.6% ($p < 0.001$) and reduced hepatic and HLN infiltration of CD2+, CD4+, CD8+ and γδ+ T lymphocytes as well as IL-4+ and IFN-γ+ cells ($p < 0.05$) [49].

Another study investigated the humoral and cellular immune responses to *F. hepatica* experimental primary and secondary infection in sheep. Western plotting results of the study revealed 12 major antigenic fractions in FhESP from 12, 15, 20, 24, 27, 28.5, 30, 41, 51, 56, 69 and 156 kDa. In contrast, the humoral response, in particular against the early recognized antigens, and the level and the duration of the FhESP-specific lymphocyte proliferative response, were reduced [50].

According to the findings of the study it can be concluded that *F. hepatica* infection induces apoptosis in the liver cells via Bax, Bcl-2 and Caspase-3 genes and it precedes necrosis. Thus, this study suggests that the induced apoptotic gene expression in hepatocytes was due to infection with *F. hepatica*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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