Original Article

Apoptosis as a Potential Target to Arrest and Survival of Hydatid Cyst

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Abstract

Background: Hydatidosis is a serious and life-threatening disease that may lead to the death of the host if diagnosed and treated improperly. Apoptosis has been investigated as a mechanism of host innate immunity in suppressing parasites and also the survival of cysts in the human body. The present study investigates the process and role of apoptosis caused by a host cell or parasite in hydatid cysts.

Materials and Methods: Survey cytotoxic effect and apoptotic mortality of hydatid-treated lymphocytes were investigated. Also, to determine the mechanism of apoptosis in host and parasite, the mean gene expressions of *Bcl-2*, *Bax*, *Caspase 3* in hydatid-treated lymphocytes, and *Fas-L* gene in the laminated-germinal layer of fertile and infertile hydatid cysts were evaluated.

Results: The viability of fertile and infertile hydatid fluid-treated lymphocytes was significantly different compared with the control group. Flow cytometry also showed apoptotic cells. *Bax* mean gene expression was significantly different between fertile and infertile treated lymphocytes. However, there was no significant difference in the mean expression of *Caspase 3,* and *Bcl-2* genes in these two groups. Although the expression of the *Fas-L* gene in infertile cysts was higher than in fertile cysts, the result was not significant.

Conclusion: It seems that hydatid cyst fluid may induce apoptosis in lymphocytes so that, hydatid cysts can escape from the immune system and stay alive. On the other hand, the results represent the possible immune path of host apoptosis against the parasite as one of the important routes in infertility of hydatid cysts.

Keywords: Apoptosis, *Bax*, *Bcl-2*, *Caspase 3*, *Fas-L*, hydatid cyst

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Introduction

Hydatidosis is a serious and life-threatening disease that may lead to the death of the host if diagnosed and treated improperly.[1] Currently, surgery is the first-choice treatment for hydatid cysts. However, it has not always been an efficient or successful modality, especially for patients with multiple cysts in different organs or those without physical suitable conditions. It may also be associated with local recurrence or secondary cysts. Fertile hydatid cyst plays an important role in causing anaphylactic reactions during cyst surgery due to protoscolex and cyclophilin antigens, antigen B, antigen 5, and elongation factor 1-β/ð. Therefore, developing in-vivo mechanisms for making cysts infertile is necessary.[2,3] However, the long survival of hydatid cysts in the intermediate host suggests that the parasite develops strategies to escape the host's immune system. In addition to the cystic fibrosis layer, which functions as a physical barrier, antigenic compounds in the cyst fluid also contribute to the parasite's escape from the host immune system.[4,5]

In recent years, several studies have been performed on the mechanisms of hydatid cyst establishment and also molecular and biological processes involved in the fertility and infertility

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of these cysts. It is proven that the bovine susceptibility to *Echinococcus. granulosus* infected eggs, most of the cysts in animals are infertile, without developing germ capsules and protoscolices. In contrast, most of the cysts in the sheep are fertile. It is hypothesized that this discrepancy may be due to the parasite strain diversity,[6] but it has been reported that fertile and infertile cysts are created in the same genotype. For example, G1 strain produced cysts in bovine, sheep, and human. Forming fertile and infertile cysts by the special strain may be relating to immune system of different hosts, which prevents the growth and development of protoscolex and makes the cyst infertile.[7]

Apoptosis has recently been studied as an important mechanism of the host innate immune system in suppressing parasites, and the parasite's defense against the host immune system and its modulation.[8] It is defined as a kind of programmed cell death characterized by morphological and biochemical changes. Two important external (death receptors) and internal (mitochondrial) paths are involved in this mechanism, and several factors play a role in each path. The enzymatic cascade of caspases and the *Bcl-2* protein family are two important members of internally programmed death. *Caspase 3* in the enzymatic cascade, pro-apoptotic protein and *Bcl-2* anti-apoptotic protein from the *Bcl-2* family play the most prominent role in apoptosis,[9] while *Fas-L* has the most important as an apoptosis inductor in the external path.[10]

Nowadays, the role of host apoptotic factors in death and the survival of different parasites such as *Echinococcus* is advert in numerous studies. Paredes *et al*.[11] found an increase in *Caspase 3* activity and deoxyribonucleic Acid (DNA) fragmentation in the germinal layer of infertile cysts compared to the fertile cysts and postulated apoptosis as one of the sterilizer mechanisms for hydatid cysts. Other studies on fertilization and infertilization mechanisms of hydatid cysts suggested an increase in the expression of Eg RAD-9 genes, apoptosis, and DNA fragmentation in protoscoleces exposed to pharmaceuticals and gamma radiation as well as in the germinal layer of infertile cysts compared to fertile ones.[12–14] Spotin *et al*.[15] used the germinal layer of fertile and infertile human cysts to evaluate apoptosis. Their findings indicated the apoptosis induction in the germinal layer of the infertile cyst. Accordingly, cystic apoptosis is regarded as a type of cellular death triggered by external factors, which leads to the self-destruction of cells. Hence, it can be a potentially efficient way of killing the protoscoleces of *E. granulosus*.

Studies on apoptosis potentials as a therapeutic target indicate the necessity of apoptosis-inducing pharmaceuticals in evaluation for parasitic disease treatment.

This therapeutic window could repress the unacceptable mortality from these diseases.[16–19] Thus, the exploration and understanding of the mechanism of apoptosis caused or controlled, by a host cell or parasite are critical. In the present study, the effect of cytotoxicity, cellular death determination, induction of apoptosis, and its mechanism in bovine HF on bovine lymphocyte cells as effective immune cells against *E. granulosus* were investigated along with the expression of the apoptotic gene in fertile and infertile hydatid cysts as potential triggers of infertility induction of cysts.

Materials and Methods

1. Sample collection

a. Hydatid cyst

The bovine liver infected with hydatid cyst provided from Fasaran Slaughterhouse, Isfahan-Iran was transferred to the parasitology laboratory of Isfahan University of Medical Sciences. Cysts were examined for the presence or absence of protoscoleces and hooks before sampling the fluid of fertile and infertile cysts. The fluids from fertile/infertile cysts were centrifuged in sterile 50 ml Falcon tubes at 2000 × g for 5 minutes. Then, clear supernatant fluid was filtered through a sterile 0.2 μm membrane and stored at −20 ºC until use. In addition, for evaluation of *Fas-L* apoptotic molecule, small pieces of laminated-germinal layer and respective adjacent normal tissue as the control were transferred in 500 μl ribonucleic acid (RNA) Later (Ambion, 76104), at −20°C until use.

b. Bovine Peripheral Blood Mononuclear Cells harvest

Blood samples were collected from healthy, hydatid cyst free 8-month-old steers in accordance with animal care regulations. Blood was diluted 1:1 with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}; 10 ml of the diluted HBSS-blood mixture gently layered a top 4 ml of the Ficoll-Hypaque (Lymphodex, inno-train, H9L6095) and centrifuged (1500 × g, at 18°C, for 30 min, without brake) (SIGMA, 3K30, USA). The layer of peripheral blood mononuclear cells (PBMCs) was collected, and washed two times in HBSS (450 g at 4°C for 5 min).

The cells were resuspended in RPMI 1640 complete and cultured in six-well tissue culture plate at a concentration of approximately 2×10^7 cells/well. After 2–3 hours incubation, supernatant of culture contains lymphocyte were collected and washed. The viability of lymphocytes are checked using trypan blue. Then, the cells were cultured in RPMI-1640 complete medium for further experiments.

2. Experimental studies

a. Cell viability assay/cytotoxicity

The viability of the cells was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). Lymphocytes were seeded into 96-well flat bottom micro culture plates at densities of 10^5 cells/ml per well. The cells were treated with the concentrations (10% and 15%) of fertile/infertile HF for 24 hours. Untreated cells were also used as negative control.

Then, the microtiter plates were centrifuged by cytocentrifuge (Hettich, UNIVERSAL 320R) and supernatant was replaced by 80 μl of the culture medium carefully. Then, 20 μl of the MTS solution (Promega, Madison, WI, USA) was

added to each well of 96-well plates and incubated in the dark for 3 hours. The absorbance of the samples was measured with an ELISA reader (Bio RAD, Model 680, Japan).[20] The data are mean values from three different experiments. MTS reduction is used to estimate cell viability at the end of the assay.

b. Apoptosis and necrosis assay

The Annexin-V Staining (BD Biosciences kit) was used to detect apoptosis and necrosis in the treated lymphocytes. To distinguish necrotic and apoptotic cells, Propidium Iodide (PI) and Annexin-V were employed. Briefly, lymphocytes (10^6 cells/ml) were treated with 10% fertile/infertile HF (optimal HF concentrations were determined by prior experiment) and incubated for 24 hours. The cells were collected by centrifugation at 350 g for 5 min and assessed by the FACS Calibur flow cytometer (BD, USA) with 488 nm excitation, for fluorescein-conjugated Annexin-V detection, and a filter >600 nm for PI detection.[21] At least 10,000 cells were analyzed per sample. The fraction of cell populations in different quadrants was analyzed using quadrant statistics.

c. Total RNA extraction and cDNA synthesis

2×10^6 fertile/infertile HF treated lymphocytes and control cells were harvested and centrifuged at 350 g for 5 min. RNA extraction was purified using kit (Jena Bioscience PP-210S), according to the manufacturer's protocol. In addition, cyst layers and adjacent normal tissue from each isolate, were squashed with liquid nitrogen, and RNA was extracted.

The quantity and quality of the extracted RNA were determined using a nanodrop (ultraviolet) spectrophotometer (WPA-Biochrom, England) at 260/280 nm. The RNA was reverse transcribed into cDNA using Revert Aid First strand cDNA synthesis Transcriptase kit (Fermentas, #k1621) according to the manufacturer's protocol.

d. Real -Time PCR

Primers for *Bcl-2, Bax*, *CASP 3*, *Fas-L* and *GAPDH* (internal control) were designed using primer blast online software [Table 1]. The experiments were conducted using the Syber Green kit (Ampliqon, A325402). For this purpose, 10 µl Master Mix, 1.5 µl cDNA, and 1 µl of each primer (10 pmol) was mixed with 6.5 µl of distilled water and placed in the Real-Time PCR (ABI, U.S.) thermocycler. The Real-Time PCR program was followed by 95°C for 10 min, and samples were amplified for 40 cycles (95°C for 15 s; 60°C for 60 s). This stage was repeated three times for each sample, and the cDNA of untreated lymphocytes and healthy liver tissue of each cyst was used as a negative control. Relative quantification of a target gene was done by comparing the expression level of reference gene *GAPDH*.

Statistical analysis

Statistical analyses were performed using SPSS Software ver 20.0 The data (MTS results) are expressed as mean ± standard deviation and were statistically analyzed using a one-sample T-test and independent T-test. The apoptotic cells were determined as the percentage of cells. To determine changes in gene expression was used One-way ANOVA. The *P* values less than or equal to 0.05 were considered significant.

Results

MTS Test Results

The metabolic activity of fertile/infertile HF treated lymphocytes was measured using one-sample T-test. The results showed a significant difference in the mean cell viability at 10% and 15% concentrations after 24 hours in the both groups (fertile/infertile HF treated-lymphocytes) compared to the control group [Figure 1].

Results of apoptosis and necrosis by flow cytometry

Cultured lymphocytes cells were incubated with 10% of fertile/infertile HF for 24 hours, and the apoptotic effect was analyzed with flow cytometry using annexin V-binding capacity methods. As seen in Figure 2 and Table 2, percentages of late apoptotic cells for lymphocyte cells incubated with the concentration of 10% fertile and infertile of HF were determined after 24 hours as 21% and 19%, respectively. In the control group consisting of lymphocyte cells that were not treated with HF, all cells, except 3% late apoptotic and 7.3% necrotic cells, were analyzed till 89% viability was reached [Figure 2c]. At the lymphocyte cells treated with fertile hydatid cyst, percentages of early and late apoptotic cells, viable cells did not exhibit

Table 1: Primer sequences and details of each gene target analyzed in bovine PBMC by quantitative real time PCR

Genes	Sequence (5'→3')	Acc No	Size (bp)
Bax	F-TTTGCTTCAGGGTTTCATCC R-CAGTTGAAGTTGCCGTCAGA	173894.1	174
Bcl-2	F-GTCATGTGTGTGGAGAGCGTC R-TCCACAAAGGCGTCCCAG	001166486.1	134
CASP-3	F-AGAACTGGACTGTGGTATTGAGAC R-TCGCCAGGAAAAGTAACCAG	001077840	124
Fas-L	F-TGTGTCTCCTTGTGATGTTTTTC R-AGATGATGCTGTATGCCTTTG	001098859.2	124
GAPDH	F- TCGGAGTGAACGGATTCG R- CATGTAGTGAAGGTCAATGAAGG	001034034.2	113

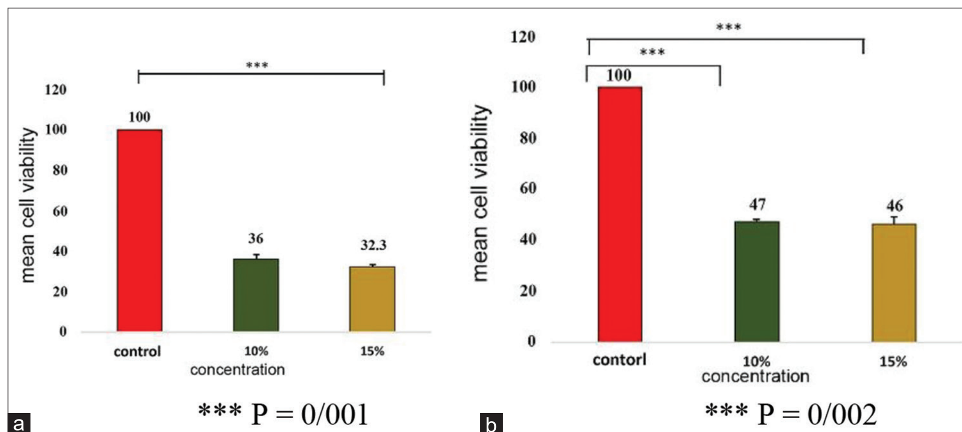

Figure 1: Comparison of mean cell viability of treated lymphocytes with concentrations of fertile (a) and infertile (b) hydatid fluid with control. Data are reported as the means ± SD

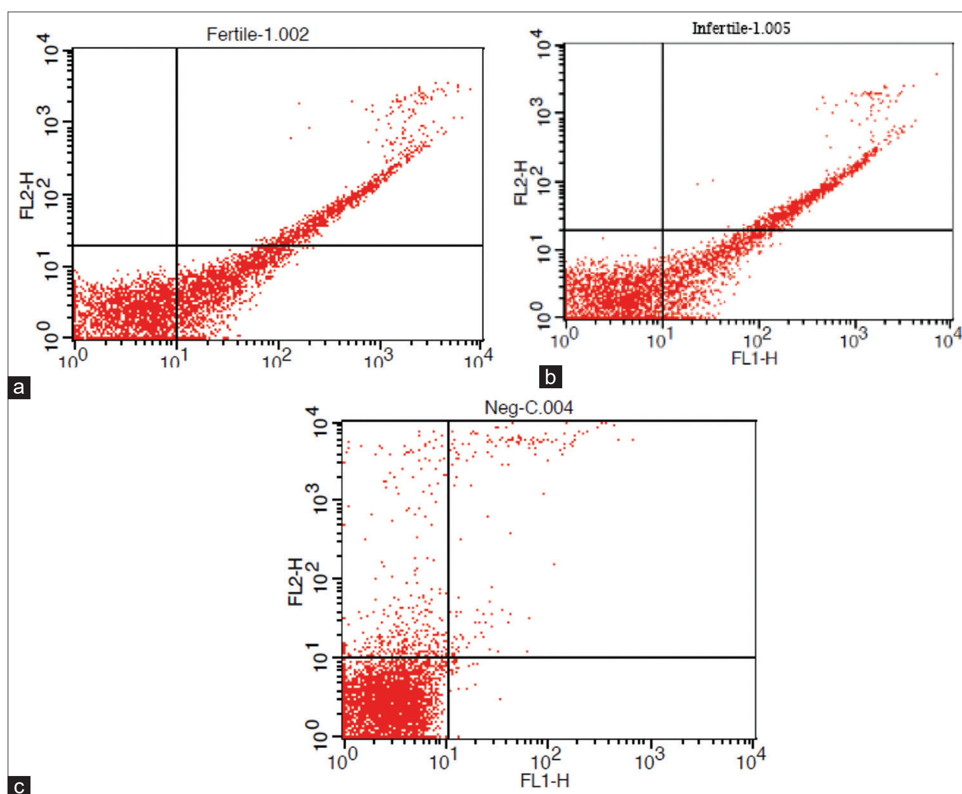

Figure 2: Measurements of Apoptosis in lymphocytes induced by fertile (a), infertile (b) hydatid cyst fluid and Control group (c) by flow cytometry

significant differences to lymphocyte cells treated with infertile hydatid cyst [Figure 2a-c and Table 2].

Real-time PCR test results

The expression of the mean *Bax* gene was significantly different between fertile and infertile treated lymphocytes (P = 0.046), [Figure 3a]. However, there was no significant difference in mean expression of *Caspase 3* and *Bcl-2* anti-apoptotic genes in these two groups, Figure 3b-c. The expression of the *Fas-L* gene in the laminated-germinal layer of infertile cysts was higher of fertile cysts, however, it was not significant [Figure 4a-c].

Discussion

Parasites develop different strategies to escape from host immune system. The *E. granulosus* has complex defense mechanisms in the larval stage improving the survival of the organism in the host body by escaping and modulation of the host's immune responses. Apoptosis has been studied recently as an important mechanism of the parasite defense against the host immune system, which can modify the immune system. Several studies have reported the toxic and apoptotic properties of HF for different types of immune cells.[13,22–25] In present study, the effects of cytotoxicity, apoptosis, and apoptosis

Table 2: Typical quadrant analysis of annexin V FITC/propidium iodide flow cytometry of lymphocytes cells treated with fertile/infertile hydatid cyst fluid

Groups	% Early apoptotic cells	% Late apoptotic cells	% Viable cells	% Necrosis
Control (untreated)	0.7	3	89	7.3
Fertile HF treated	28	21	51	0
Infertile HF treated	27	19	54	0

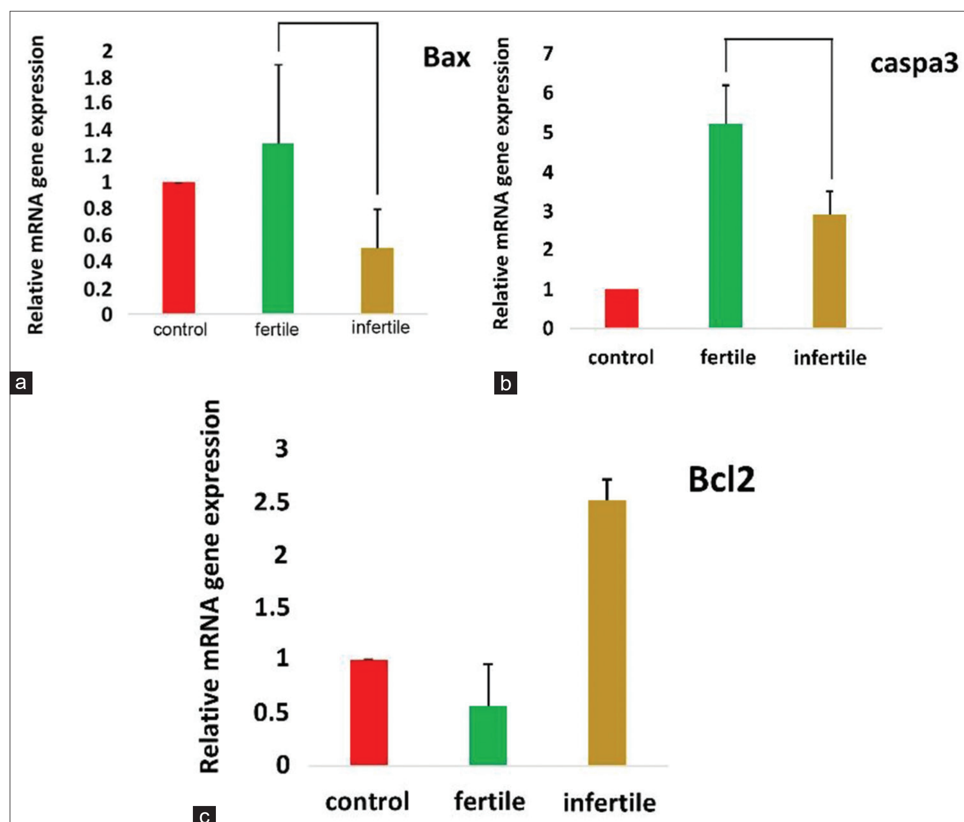

Figure 3: A: Comparison of *Bax* (a), *Caspase 3* (b), *Bcl2* (c) genes expression mean in lymphocytes treated with fertile/infertile cyst fluid and control group

induction mechanism of bovine HF on lymphocyte cells were investigated as effective immune cells against *Echinococcus* species. Our findings showed that HF can reduce the viability and metabolic activity of lymphocytes. Our results also showed induces apoptosis in these cells by increasing the expression of the *Bax* gene and *Caspase 3* enzyme and decreasing the expression of *Bcl-2* anti-apoptotic genes.

The effect of HF on the viability of some cell lines and its apoptosis induction property has been investigated in the research literature. The findings are consistent with the results of the present study. Yin *et al*.[25] showed that HF reduces the cytotoxic effect of natural killer cells by reducing the expression of NKG2D, the key stimulator cytokine for killer cells. Using the MTT & Flow cytometry method s, a number of researchers examined the viability of macrophages treated with HF. Their findings showed that HF has the potential for reducing the macrophages' viability from the apoptotic path.[23,24] The MTS results of the present study indicated that there is a significant intra/intergroup difference in the mean viability of the lymphocytes for groups treated with fertile and infertile hydatid cyst fluid and also the control group.

Our findings in line with research literature show that the culture system provides a simple assessment of the cytotoxic effect of hydatid fluid. On the other hand, HF appears to contain molecules inhibiting immune cells. These molecules, created by the parasite, may be gradually released into the vicinity of the cyst, inhibiting and killing immune cells such as macrophages, lymphocytes, and natural killer cells of the host .[15] This can be an explanation for the parasite's escape from the host immune defense.

Flow cytometry results in this study, showed that the percentage of viable cells in the control group is higher than the treatment groups. This is consistent with the results from the MTS test. Also, increased $Annexin^+/PI^-$ and $Annexin^+/PI^+$ cells population in the treated groups compared to the control group suggested apoptosis as the cause of lymphocyte cells' death.

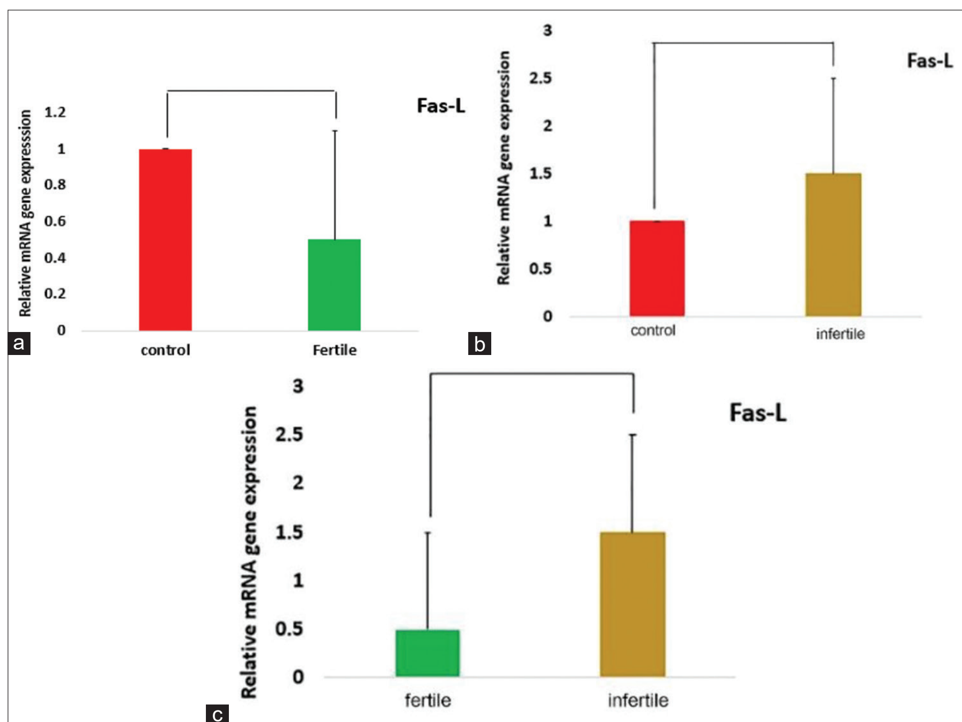

Figure 4: (a): Comparison of *Fas-L* gene expression mean in fertile and healthy tissue groups. (b): Comparison of *Fas-L* gene expression mean in infertile and healthy tissue groups. (c): Comparison of *Fas-L* gene expression mean in fertile and infertile groups

Since the HF has been added without any apoptosis inductive agent, it seems that HF contains lymphotoxin with the ability to directly inhibit the function of bovine lymphocytes and cause their apoptosis.

To determine the mechanism of apoptosis, the mean expression of the *Bax* pro-apoptotic gene, *Bcl-2* anti-apoptotic gene, and *Caspase 3* enzyme were measured playing a key role in the mechanism of apoptosis. Since toxins are activators of the internal pathway, it appears that hydatid cyst fluid induces lymphocyte apoptosis through its toxin effect in the mitochondrial pathway. The increased expression of *Bax* gene and decreased expression of *Bcl*-2 anti-apoptotic gene by modification and increasing the permeability of mitochondrial membrane followed by Caspase cascade activation and increased expression of *Caspase* 3 lead to apoptosis of lymphocytes.

Our findings are consistent with the results of Spotin *et al*.[15] In studies by Justin *et al.* and Macintyre *et al*., the mitotic activity and alterations in lymphocytes and dendritic cells' membranes exposed to different concentrations of HF were investigated in the culture medium. The results indicated the cytotoxic effects of HF in suppressing the cellular S phase activity along with changes in lymphocyte membranes and the increased CD86 and MHCII on dendritic cell membranes. It also showed the increased CD38 and CD25 and decreased CD28 on T-cell membranes which may suggest the apoptosis or anergy of T-cells due to exposure to hydatid fluid. However, the infertile HF had a lower inhibitory effect than the fertile HF.[26,27] The results of the present study also showed that infertile HF has a lower cytotoxic and apoptotic effect than fertile HF. The fertility of the cyst is not a necessary condition for the toxin or apoptotic effect of HF because the infertile cyst fluid also reduces the viability of lymphocytes and leads to their apoptosis. However, it can be shown that the potential for balance and modulation of the host immune system by the secretion of a hydatid cyst is a characteristic of the parasite's internal environment that may organize the response against the host.

Our results also show that the viability and metabolic activity of lymphocytes treated with infertile HF were higher, while induced apoptosis was lower. Therefore, it appears that the molecules in the two cysts are different. Moreover, antigens secreted by protoscoleces in the fertile cysts may induce higher apoptosis in the host lymphocytes. Several studies demonstrated the influence of hydatid cyst fluid and protoscoleces on reducing the growth of tumor cells by increasing cellular death and inhibiting T-cell proliferation.[28-31]

These studies indicate that hydatid cyst fluid molecules and protoscoleces can trigger apoptosis in host immune cells. Therefore, one of the reasons for the survival of the *E. granulosus* in the host is the presence of essential proteins in the cyst fluid. Hydatid cyst fluid has the potential to regulate the innate immune response of the host to this parasite. HF is a complex mixture of compounds derived from the host and the metabolic activity of the parasite. Thus, further studies are required to identify molecules and compounds in fertile and infertile cysts in different hosts.

To identify the molecular and biological mechanisms involved in the fertility and infertility of hydatid cysts, the present study detected significant changes by comparing *Fas-L* gene expression in fertile/infertile cysts and healthy host tissue. It demonstrated that *Fas-L* expression as an inducer of apoptosis in the laminated-germinal layer of infertile cysts was higher compared to the fertile and control group.

In our study, the results from laminated-germinal genotyping of all fertile and infertile cysts was G1.[32] Thus, the fertility and infertility of cysts are independent of parasite genotype and reliant on the host immune system. The results of this study are in line with the findings of Paredes and Spotin.[11,15,33]

Conclusion

Although surgery is the first-choice line for the treatment of hydatid cysts, this method has not always been beneficial or effective in patients with multiple cysts in different organs or those with no suitable physical conditions. In some cases, it has also led to the local recurrence or secondary cyst formation.[3] More investigations into the mechanism of protoscoleces apoptosis will advance a new treatment theory for hydatid disease in the future. Thus, it seems necessary to develop mechanisms leading to the infertility of cysts in the host.

Declarations

Ethics approval and consent to participate:

The Ethics Committee of the Isfahan University of Medical Sciences confirmed that the research proposal and procedures were in accordance with the animal ethical standards (IR.MUI.MED.REC.1399.678).

Consent for publication

Not applicable

Availability of data and materials

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

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Conflicts of interest

There are no conflicts of interest.

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