Immunohistochemical Evaluation of Apoptotic Proteins Expression in Liver and Spleen after Treatment of Cystic Echinococcosis: An Experimental Study

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Summary

The cellular immune response and apoptotic pathways are closely related dose dependent responses against *Echinococcus granulosus* antigens. The aim of this study is to evaluate the cellular expression of Bax, Bcl-2 and Caspase-3 in both liver and spleen of experimentally cystic echinococcosis mice treated with Oxfendazole, Oxfendazole +Praziquantel, Oxfendazole +Albendazole and Albendazole +Praziquantel of experimentally mice model of cystic echinococcosis. After 2 months of treatment, mice were sacrificed and both liver and spleen were processed for immunohistochemistry staining protocol using specific primary antibody against mouse Bax, Bcl-2 and Caspase-3 proteins. The results showed that *E. granulosus* infection able to induce apoptosis in both liver and spleen tissues after induction of cystic echinococcosis mice model, treatment with Albendazole in combination form gives better results because of attenuation of apoptosis pathway and restore of normal cellular behavior. In conclusion, this study describes the involvement of apoptosis in the pathogenesis of cystic echinococcosis.

Key words: *Apoptosis, liver, Spleen and cystic echinococcosis.*

Introduction

Cystic echinococcosis (CE) is one of the most chronic helminthic wide spread zoonotic disease in many countries caused by metacestode of *Echinococcus granulosus*[1]. The interplay between parasite and host cells have been described ensuring survival of parasite[2]. CE can persist chronically in the affected organ mainly liver evading host immune responses and induction of apoptosis in lymphocytes[3].

Apoptosis or programmed cell death is an important cellular mechanisms ensuring cellular homeostasis[4]. Studies showed that fertile hydatid cyst containing protoscolex were able to induce apoptosis in the adjacent cells of the host[5]. Thus, *E. granulosus* is capable to modulate host immune responses such as the interference with modulation of dendritic cells maturation [6], and induction of a non-protective Th2 cell response by AgB[7]. Apoptosis is another proposed mechanism[8] that provides suitable environment for survival of the cyst by inducing apoptosis in the host immune cells. Several factors play role in apoptosis, but the caspase enzymes and Bcl-2 family are the two main families in this process. The first is a cascade of enzyme, of which Caspase-3 is the most important member affecting the lymphocyte apoptosis. The second is Bcl-2 family, a set of cytoplasmic proteins members that regulate apoptosis[9] like Bcl-2 and Bax proteins. While Bcl-2 proteins inhibit apoptosis, Bax counteracts this [10]. Central enzyme of apoptosis cascade is a proteolytic system involving a family of proteases called caspase. Among these enzymes Caspase -3 plays a central role in the apoptosis process[11].

The current study aimed to investigate the protein expression of Bax, Bcl-2 and Caspase 3 in liver and spleen of mouse model of cystic echinococcosis under

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treatment with different modalities of anthelminthic drugs.

Methods

Experimental design:

This in vivo mouse model experiment for cystic echinococcosis were approved by College of Pure Sciences (Ibn AL-Haitham)/ Baghdad University. This experiment was done in Animal housing of the college. Balb/C male mice were housed inside cages according to the instructions.

Collection of hydatid cysts samples and isolation of protoscolices: Hydatid cysts from lungs and livers of infected sheep carcasses collected in AL-Sadir slaughter in Baghdad. These cysts were stored and processed to separate the protoscoleses from the fluid[12].

Preparation and administrations of drugs:

Three drugs were used in this study, Albendazole (ABZ) 10mg/kg, Praziquantel (PZQ) 40mg/kg and Oxfendazole (OXF) 30 mg/kg. These drugs were dissolved in distilled water and stored at 4°C for no more than 24 hours. Drugs were used about two months in single form or combination with others as the following:

OXF treated group, OXF+PZQ treated group, OXF+ABZ treated group and ABZ+PZQ treated group. Additionally, not infected group served as negative control (NC) group while none treated group served as positive control group (PC).

All drugs were given after four months of experimental infection with 2000 protoscolices

(I/P) in accordingly to the group of study as single dose orally at early morning for two months.

Immunohistochemistry staining of BAX, Bcl-2 and Caspase -3 proteins in liver and spleen sections:

Animals were sacrificed by cervical dislocation, then examined their internal organs (such as Liver, spleen, lung, stomach intestine and etc.) of mice were removed under sterile conditions by abdominal incision. Both liver and spleen were washed in distilled water and stored in 70% ethanol and processed as paraffin embedded tissue blocks.

Sections of 5 micrometer were obtained and mounted on positive charge glass slides. Before labeling, sections were deparaffinized in xylene and rehydration by graded series of ethanol baths. Endogenous peroxidase activity was blocked with peroxidase block. After washing, sections were then incubated in protein block for 20 minutes at room temperature.

100 microliters of diluted primary antibody against Caspase -3 (orb382909),BAX (orb378567) and BCL2 (orb10173) was added on tissue section for 60 minutes. After washing, visualization steps achieved by Rabbit IgG SABC Kit (orb90444) using DAB Chromogen Kit (orb219876) and counterstained by hematoxylin.

Data analysis

Results of IHC test were evaluated by applying a semi- quantitative assessment: each slide was counted under light microscope for three times at x400 magnification and about 5 fields were randomly selected in each round. Thus, the number of immunolabeled cells was counted in 5 fields under a fixed focus for each slide and value of mean for positive count in total number of all counted cells (each tissue section) for each sample group. Data were expressed

as mean ± standard deviation (SD) and Analysis of Variance (ANOVA) test was used for differences between groups. Values $p \leq 0.05$ was regarded as statistically significant.

Results

This study investigates the apoptosis regulating proteins expression in liver and spleen of mice before and after treatment with different drugs. After induction of cystic echinococcosis in mice, the apoptosis proteins (Bcl-2 and Bax) were elevated in both liver and spleen tissues. Similarly, Caspase -3 was highly elevated in comparison with negative group (Fig. 1).

Restoration of apoptotic protein expression after treatment of cystic echinococcosis:

In the current experiment, it's clearly shown that apoptosis proteins were reduced in both liver and spleen tissue after four months Cystic Echinococcosis and two months of treatment of cystic echinococcosis with different treatments as shown in Table 1.

Bax protein was still elevated in liver tissue under treatment with OXF alone or in combination with PZQ or ABZ, and ABZ with PZQ. But it was reduced in spleen tissue under treatment with OXF in combination with PZQ or ABZ as shown in Fig 1

Caspase -3 protein expression also reduced after two months of treatment in combination treatments in comparison with non-treated group

Table1: Comparison of Bax, Bcl-2 and Caspase- 3 proteins in liver and spleen in study groups.

liver	**NC**	**PC**	**OXF**	**OXF+PZQ**	**OXF+ABZ**	**ABZ+PZQ**
Bax	15.4±4.7	27.83±7.83 A**	17.23±8.38 ANS, B*	18.03±6.94 ANS, B*	16.94±7.03 ANS, B**	18.2±6.02 ANS, B**
Bcl2	5.03±2.9	8.93±1.9 A**	6.92±3.9 ANS, BNS	6±2.3 ANS, BNS	8.9±2.6 A**, BNS	7.9±2.8 A**, BNS
Caspase3	15.28±7.44	32.3±12.84 A**	26.84±11.84 A**, B*	14.32±9.44 ANS, B**	10.5±7.54 ANS, B**	12.44±8.54 ANS, B**
Spleen	NC	PC	OXF	OXF+PZQ	OXF+ABZ	ABZ+PZQ
Bax	7.93±2.8	18.92±10.2 A**	12.2±8.3 ANS, B**	8.93±3.9 ANS, B**	10.23±5.3 ANS, B**	11.2±3.9 ANS, B*
Bcl2	6.8±3.9	12.2±6.9 A**	12.9±7.2 A**, BNS	4.8±2.1 ANS, B**	3.9±1.8 A*, B**	6.7±2 ANS, B**
Caspase3	17.29±7.4	38.92±13.4 A**	28.32±10.84 A**, B*	17.93±10.2 ANS, B**	14.33±8.99 ANS, B**	14.2±10.4 ANS, B**

A: statistically significant difference from Negative control (NC), B: statistically significant difference from positive control (PC).* Significant differences on ($p \leq 0.05$). **Significant differences on ($p \leq .0.001$). NS: No significant ($p>0.05$).

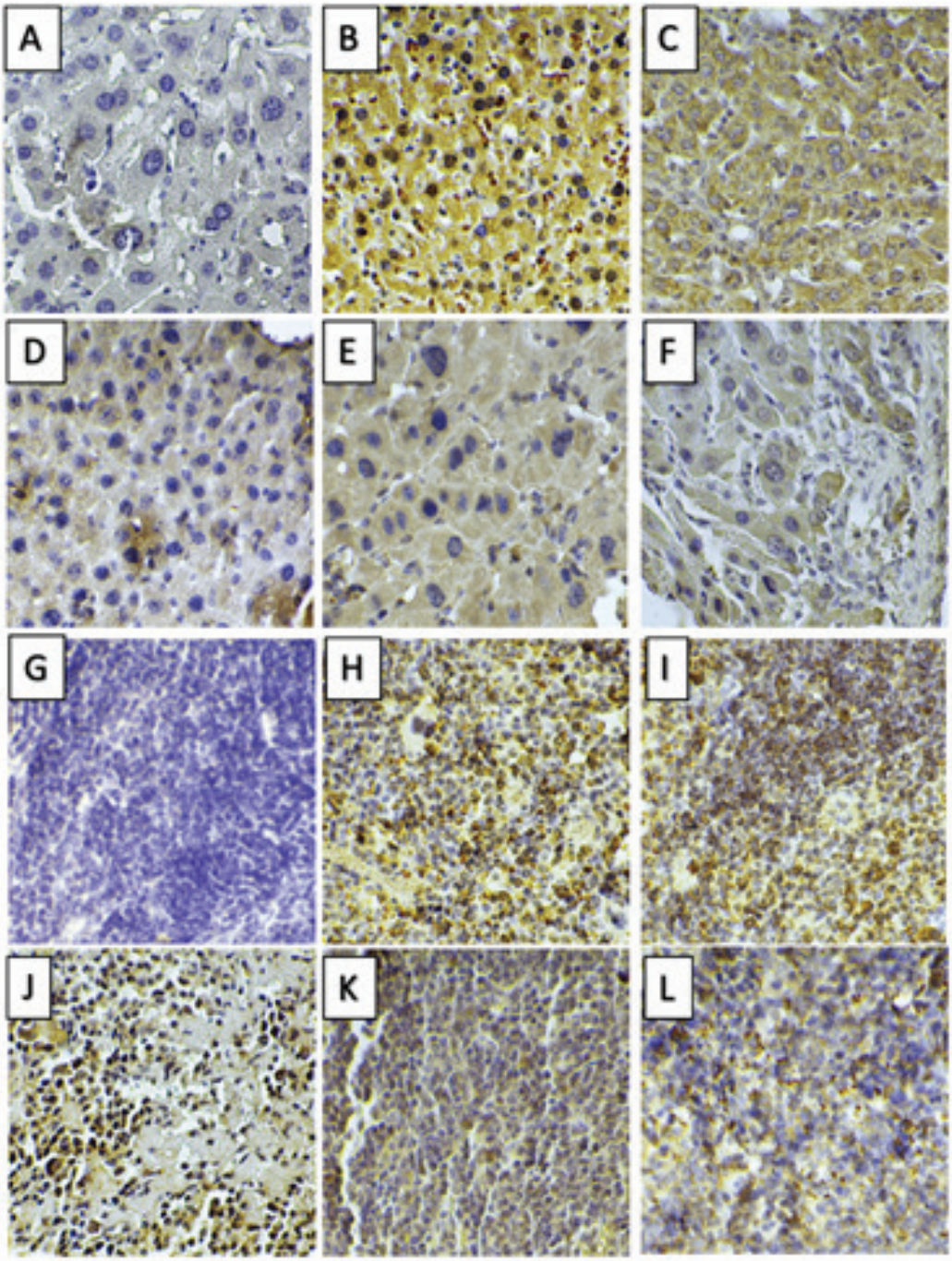

Figure 1: Immunoperoxidase staining of Bax protein expression in liver and spleen in study groups using polyclonal rabbit anti- Bax (5pg/ml). (A-F): Bax expression in spleen tissue, (A) control negative group with low expression, (B) positive control group without treatment showing high expression of Bax protein, (C) OXF treated group, (D) OXF+PZQ treated group, (E) OXF+ABZ treated group and (F) ABZ+PZQ treated group. (G-L): Bax expression in liver tissue, (G) control negative group with low expression, (H) positive control group without treatment showing high expression of Bax protein, (I) OXF treated group, (J) OXF+PZQ treated group, (K) OXF+ABZ treated group and (L) ABZ+PZQ treated group.

Discussion

The effect of *E. granulosus* infection on apoptotic proteins expression in liver and spleen were not yet studied. However, this is the first study that report the immunohistochemical expression of Bcl-2, Bax and Caspase 3 protein expression in both liver and spleen of mouse model of cystic echinococcosis after treatment with different treatments.

In the current experiment, an increased apoptotic protein in liver and spleen after induction of infection suggesting a higher activity of apoptosis pathway mediated by parasitic antigens. This inclination was argued by other studies proposed that the parasite release antigens that modulate apoptosis in host cells. This was evident by mitogenic activity of protoscoleses on regulation of growth in leukocyte cell line[8,13].

Our results was argued by Amirmajidi, *et al*., 2011 whom reported that apoptosis was higher in lymphocytes after treatment with fertile hydatid cyst fluid compared with control cells. Furthermore, Bcl-2 mRNA as anti-apoptotic protein was reduced accompanied by increased Bax protein as pro-apoptotic protein in in this model, Additionally, increased caspase-3 expression was also higher compared with control group[3].

Another studies, Macintyre *et al*.,2001 found that hydatid fluid induce cell proliferation with enhanced expression CD25 and CD38 on human peripheral blood lymphoblast and reduced CD28 (and other co-stimulatory molecules) resulting in anergy or apoptosis[14]. Also, Li *et al*.,2003 showed that apoptosis was significantly higher in T-cell after infection, this might be due to release of inducers of apoptosis such as antigens[15] thus, fertility of cyst is an important factor for survival of the cyst in the host's body[16].

The induction of apoptosis has significant advantage for the *Echinococcus granulosus* infection as a fertile stage induce apoptosis in the leukocyte infiltration toward the newly growing cyst[3].This strategy more important in the early stage of infection ensuring minimal concentration of leukocytes as an immune suppression mechanism.

This study clearly highlighted that two months of treatments were restored the expression of Bcl2 (anti-apoptotic protein) and reduce Bax and caspase- 3 (pro-apoptotic proteins) in both liver and spleen of mice model. However, it might be due to scolicidal activity of treatment against parasite leading to reduction of parasite load that ultimately reduce the inducers of apoptosis of host cells[17]. Similarly, this explanation was clearly demonstrated the scolicidal and apoptotic activities of albendazole against *E. granulosus*[18,19] or in liver tissue in combination with Chinese herbs (*Sophora moorcroftiana* alkaloids)[19,20].

In conclusion, this study describes the involvement of apoptosis in the pathogenesis of cystic echinococcosis. Using of Albendazole in combination form gives better results because of restoration of apoptosis pathway.

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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