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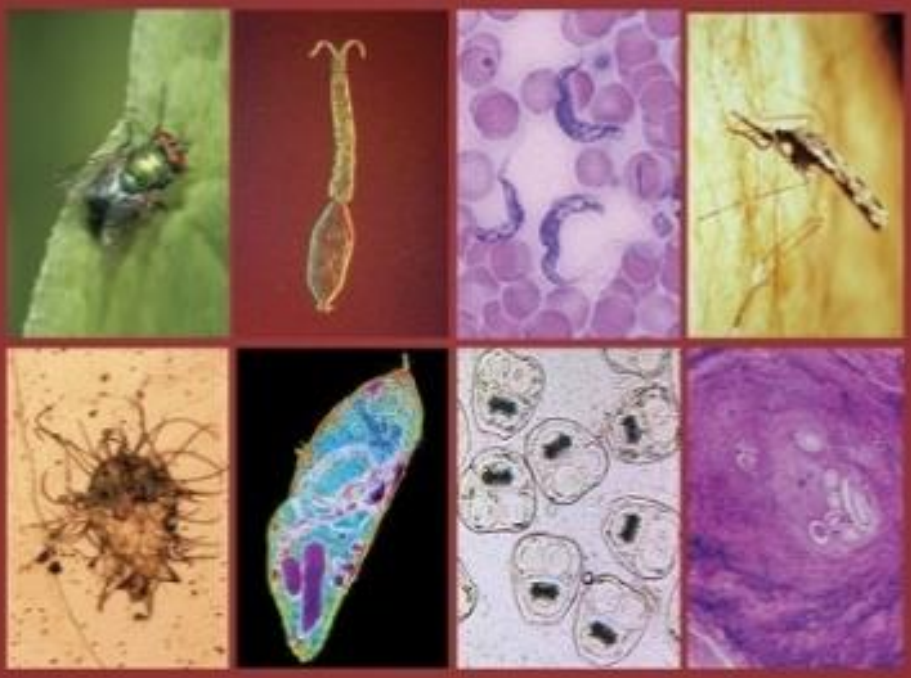

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Effect of Interferon-*α* Immunotherapy, Combined with Albendazole on The Integrity of Hydatid Cysts' Germinal Epithelium: Experimental Study

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ABSTRACT

Medical treatment for human hydatidosis may be the only therapeutic opportunity for those facing problems to perform surgical interventions or because the infection is widespread or anatomically inaccessible. Medical treatment depends on albendazole and mebendazole which are the only drugs that can effectively inhibit the growth of *Echinococcus* larvae, but unfortunately, failure of such medicinal strategy is repeatedly documented. Immunotherapeutic agents such as cytokines are one of the therapeutic modalities that can enhance the efficacy of albendazole by promoting an effectual immune response. Therefore, the aim of the current study was to assess the effect of INF- *α* immunotherapy, in combination with albendazole on the integrity of the germinal layer of hydatid cysts, using a mice model. 4',6-Diamidino-2-phenylindole (DAPI) blue fluorescent staining was used to reflect the level of regeneration within the germinal epithelium. The cysts related to a group of mice treated with albendazole showed a significant reduction in number (73.02%), compared to the control group ($P<0.05$). Yet the group that received combined therapy showed dramatic changes and the best reduction rate (94.29%). DAPI stain reflected a vital sign, denoting cellular damage within the germinal layer with extremely low expression 1.8±2.1 in the group treated with combined therapy, in comparison to control 49.6±11-7 ($P<0.05$). While albendazole reported an expression of 17.04±5.3, denoting its ineffectiveness in radically destroying the germinal layer. Administration of INF- *α* as adjuvant immunotherapy, in combination with albendazole is recommended for medical therapy of hydatid disease, especially in cases where surgical interventions are difficult or represent a serious risk to the patients. Further study concerning the uses of different treatment doses and durations is highly recommended to confirm the effect of the combined immunotherapy with the anti-parasitic drug.

INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic infection affecting chiefly livestock and transmitted mainly by canines (Tamarozzi *et al.,* 2020). Although hydatidosis is not a widespread disease among humans, still it is reported among humans in sheep grazing areas and is considered a serious public health problem in Central Asia and China, South America, and Mediterranean countries, including Egypt (Ito, 2017). It can be transmitted to human subjects through consuming contaminated food with *Echinococcus* eggs or during playing with infected dogs, causing cystic larval stages within variable organs. Yet, hepatic location is the most predominant site, reaching up to 70 % of cases (Ali *et al.,* 2012; Malekifard and Keramati 2018).

Treatment options for CE include active surveillance, anti-parasitic medications and surgery, including percutaneous surgical intervention which is known as PAIR technique (Larrieu *et al*., 2018). Medical treatment depends on the long course administration of two benzimidazoles; albendazole (ABZ) and mebendazole (MBZ), which are the only drugs that can effectively inhibit the growth of *Echinococcus* larvae (Vuitton *et al.,* 2016; Siles-Lucas *et al.,* 2018). This medical treatment is the best alternative for some patients who have missed the optimal time of surgery or for those whose parasite lesions have already been resected (McManus *et al*., 2003; Brunetti *et al*., 2010), but unfortunately, albendazole is not efficient in some cases (Brunetti *et al*., 2010). In addition, about 40% of the cases don't show favorable responses to treatment with benzimidazole (Ceballos *et al*., 2015). As a result of this questionable outcome, investigators are committed to finding more effective alternatives for the radical treatment of echinococcosis.

Significant progress has been made in both *in vivo* and *in vitro* trials in the laboratory and clinical applications (Nicolao *et al.,* 2014; Naseri *et al*., 2016; Liu *et al.,* 2018; Ma *et al*., 2020). Combinations of drugs have become an effective strategy in the treatment of echinococcosis (Wang *et al.*, 2022). It seems that immunotherapeutic modalities such as cytokines are one of the therapeutic components that can enhance the efficacy of albendazole by promoting protective immune responses (Zhang *et al*., 2012). IL12 and IFN-γ are two cytokines with stronger parasiticidal activity and enhance immune responses against secondary echinococcal cysts in mice when co-administrated with ABZ (Rahdar *et al*., 2020). In this work a trial was done to study the effect of exogenous commercially available interferon (INF- α), in combination with albendazole to study their impact on the integrity of the germinal layer of hydatid cysts, using a mice model.

MATERIALS AND METHODS

Experimental Animals:

Following ethical and institutional guidelines, this study comprised 21 laboratory-bred Swiss albino mice weighing 25-30 grams, kindly provided by the Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Animals were maintained at the animal house of TBRI until the end of the experiment. They were divided into 3 groups according to the experimental design; Group I (infection control): infected without medication. Group II: infected, treated with albendazole alone. Group III: infected, treated with albendazole and Interferon α.

Infection:

According to Rahdar *et al*., (2018) with modification, fertile hydatid cysts were collected from infected organs of Egyptian camels. Under the sterile condition, the cysts were opened and cyst fluid was aspirated and then protoscolices were collected after centrifuging and were rinsed 5 times with Gibco Roswell Park Memorial Institute (RPMI) 1640 medium. Penicillin-streptomycin 500 units/ml was added for inhibiting bacterial growth. To confirm viability, the protoscolices were investigated by 0.1% eosin vital staining.

About 95% protoscolices viability was used for infecting BALB/c mice and 1000 protoscolices were intraperitoneally injected/ mouse for induction of infection.

Medications:

Mice received the proposed medications, 4 months after infection. Lyophilized powder of Egyferon, alpha-interferon 2b, (3 million I.U/vial) [Nile Company for *Pharmaceuticals* & Chemical Industries, El-Ameria, Cairo, Egypt]. The precise amount of the lyophilized powder was dissolved in phosphate-buffered saline (PBS). The dose was adjusted to obtain a drug dose of 3000 I.U. per mouse per injection, given intraperitoneally once weekly for 7 successive doses. Albendazole, 150 mg/kg/day was given orally for 10 days. One month after completion of the treatment, mice were euthanized and dissected to collect the cysts from the peritoneal cavity. The size and number of the collected cysts were estimated in addition to investigating cyst viability.

DAPI Is Used Mostly in Fluorescence Immune Staining:

According to Atale *et al.* (2014) and following the manufacturer's instruction [Sigma-Aldrich St. Louis, Missouri, USA], 4',6-Diamidino-2-phenylindole (DAPI) blue fluorescent staining was used to preferentially stains dsDNA, reflecting the degree of cellular regeneration within the germinal layer of the examined hydatid cyst following a fixation on charged slides. Briefly, the sections were washed with phosphate-buffered saline solution (PBS). Working stock solution (50 μg mL^{-1}) was prepared by diluting the DAPI stock solution in PBS solution to allow binding to the cellular DNA. The sections were then incubated with the stain for 2–5 minutes. Sections were then washed twice with PBS and excess buffer was subsequently drained out. Sections were mounted and then covered with coverslips and slides are examined under the fluorescence microscope [Olympus. Global, Tokyo, Japan]. Reading was done using absorption maxima at a wavelength of 358 nm (ultraviolet) and the emission maximum is at 461 nm (blue), thus DAPI was excited with ultraviolet light and is detected through a blue/cyan filter. Optical density was estimated and subsequent area % of the local expression was calculated according to the software analysis for all study groups. For statistical analysis, Fisher exact test and chi-square were used and P. value <0.05 was considered for significant differences.

RESULTS

The cysts related in both groups treated with albendazole and combined immunotherapy + albendazole showed a dramatic reduction in size and number, compared to the control group ($P<0.05$). The size of the cysts, obtained in the control group was 13.2mm ±3.9. In group II, the size was significantly reduced (7.3mm±3.2), while in group III the size was dramatically reduced (2.4mm ±1.3) and the difference was statistically significant ($P\leq0.05$). As regards cyst number, in group I, cyst number reached 456±32, while in group II the number reached 123 ±19 and in group III the number was significantly reduced to 26 ±4 with statistically significant differences between groups ($P\leq0.05$). The group that received combined therapy showed the best results and the reduction rate reached 94.29%, while the albendazole group achieved 73.02%. DAPI stain succeeded to reflect a vital sign denoting cellular regeneration in variable groups with extremely very low expression in the group treated with combined therapy (1.8±2.1), in comparison to the control group (49.6±11.7) and group treated with albendazole (17.04±5.3) ($P<0.05$) (Table 1 and Fig. 1).

Table 1: Conditions of the cysts in different groups

Groups	Conditions of the cysts			
	Cyst size ±SD (mm)	**Cyst number ±SD**	**Reduction rate**	**DAPI expression**
Group I (infection control):	13.2±3.9	456±32		49.6±11.7
Group II: infected and treated with albendazole alone. Group	7.3±3.2	123±19	73.02%	17.04±5.3
III: infected treated with albendazole and Interferon	2.4±1.3	26±4	94.29%	1.8±2.1

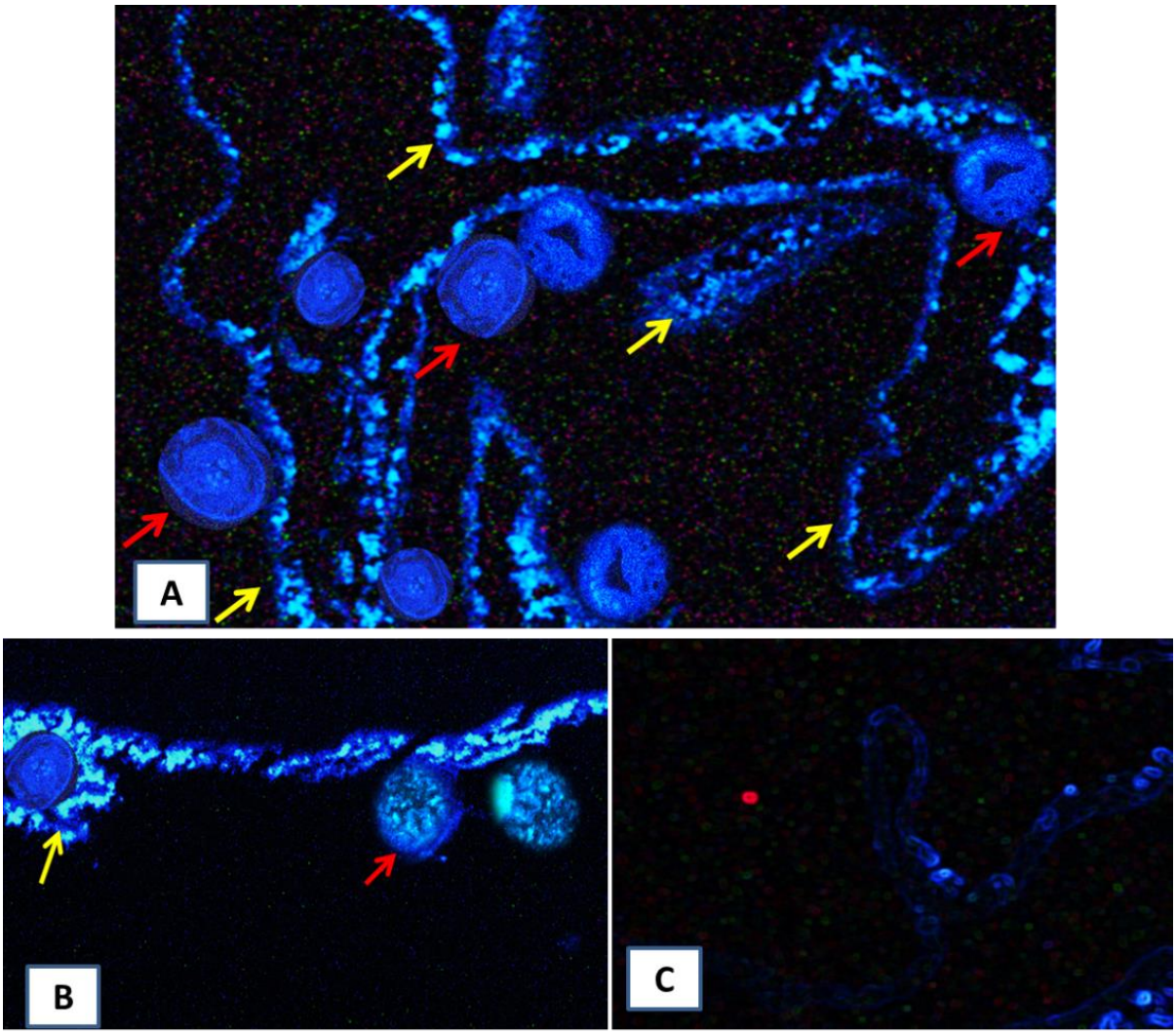

Fig. 1: Photographs represent results of DAPI nuclear stain in different study groups. **A**; control infected non-treated group shows the maximum expression level. Notice the extensive bluish discoloration within the germinal layer (yellow arrows), reflecting the healthiness of the germinative layer with numerous scolices (red arrows). **B**; group treated with albendazole only shows moderate expression with some dead scolices, while the germinal layer shows signs of nuclear regeneration. **C**; group treated with combined therapy shows very minimal expression without features of nuclear regeneration, possibly indicating complete destruction of the germinal layer.

DISCUSSION

Although there are current medical treatments for hydatid disease, such choice has repeatedly failed, and therefore the medical team is often referred to surgical options, especially if the situation calls for rapid intervention. However, there are many cases that are difficult to access surgically or that affected multiple organs, thus requiring another unconventional treatment strategy (Rivero-Lezcano, 2008). In this study, the use of INF- α was successful as an adjuvant immunotherapeutic agent, combined with albendazole to affect badly the integrity of the hydatid germinal layer and reach a reduction rate of 94.29% within the infected animals, and thus achieving a superior effect over albendazole monotherapy. Wang *et al.* (2022) reported that combined therapy can enhance the synergistic effect of the joined drugs and reduces the occurrence of drug resistance. They added that through the combined therapy, the individual dose can be reduced, thus reducing the side effects.

In fact, the strength of hydatid cysts, their viability and their ability to expand is mainly related to their inner nucleated germinal layers which germinate the protoscolices. Destruction of this layer by any of the known methods of treatment is the basis for the complete eradication of this serious parasitic stage. The survival of some of these nuclei within the germinal layer can with time, recover and produce scolices again, causing treatment failure (El saftawy *et al*., 2021). Thus**,** treatment options must have great efficiency in damaging this vital layer.

In this work, 4',6-Diamidino-2-phenylindole (DAPI) staining was used to quantitatively estimate the level of DNA within the nucleated germinal layer, reflecting the degree of vital capability in such layer. Its selectivity for nucleic acid genomic materials with high cell permeability permits effectual staining of cellular nuclei with little background noise from the cytoplasmic contents. In fact, it is a classic nuclear stain for immunofluorescence microscopy and an essential component of a high-content screening tool necessitating cell-based DNA quantitation. DAPI stain can be used for both fixed and living cell staining, though the concentration of the stain needed for living cell staining is generally much higher than for fixed cells (Atale *et al*., 2014). The high expression means the presence of excessive cellular epithelium as reported in this study within the control infected non-treated group. Extremely low expression was reported in this study with the group treated with combined therapy, indicating the success of such treatment option to damage the germinal epithelium.

Concerning immune response, it is well known that hydatid disease is under the predominance of the anti-inflammatory T helper cell population (Th2) (Bayraktar *et al.*, 2005). This is possibly the reason behind its presence inside the body organs for long periods of time, protected from the immune system. Perhaps with the help of a vital element as the pro-inflammatory Th1 mediator as interferon along with the recommended medication, albendazole can eliminate such serious life-threatening infection.

INF- α has been used before in many serious infections such as viral hepatitis (Zhang *et al*., 2014), asthma (Desai and Brightling, 2012), bacterial infection (Khamaganova *et al*., 2011), inflammatory diseases (Mullen *et al*., 2014) as well as some parasitic infection as visceral leishmaniasis (Ghosh *et al*., 2013 & de Assis Souza *et al*., 2013). Lesions related to *Leishmania major* showed a dramatic recovery after promoting Th1 cytokines and suppressing those related to Th2 (Heinzel *et al.*, 1993). Moreover, the Administration of pro-inflammatory cytokines to *Schistosoma mansoni* experimentally infected mice resulted in a reduction of egg-induced

pathologic alteration by inhibiting granuloma formation (Wynn *et al.*, 1994).

In fact, Th1 cytokine as interferon can stimulate a protective immune response via activation of macrophages and NK cells thus destroying the infectious agents. Low levels of interferon were investigated in the case of alveolar hydatidosis by Emery *et al.* (1996), who reported a high parasitic burden, with such defect. When Liance *et al*. (1998) used interferon to treat the alveolar hydatid animal model, they noticed a dramatic reduction in parasitic load. Another pro-inflammatory cytokine was used by Emery *et al.* (1998) in the treatment of hydatid diseases which is IL12. The authors recommended its use with albendazole, not only for treatment but also for the prevention of alveolar hydatidosis.

In conclusion, the administration of interferon as an adjuvant immunotherapeutic agent combined with albendazole succeeded to destroy the regenerative power of the hydatid germinal epithelium. Thus, the use of such a combination is recommended, especially in cases where surgical interventions are difficult or represent a serious risk to the patients. Further study concerning the uses of different treatment doses and durations is highly recommended to confirm the effect of the combined immunotherapy with the anti-parasitic drug.

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