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In vivo activity of albendazole in combination with thymol against *Echinococcus multilocularis*

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Highlights

- The efficacy of the combination ABZ/thymol on mice infected with *E. multilocularis* metacestodes was demonstrated.
- Combination of the two compounds showed the maximum anti-parasitic effect.
- Drugs combined caused dramatic reduction on cyst weight and severe damage on protoscoleces and metacestodes.

ABSTRACT

Human alveolar echinococcosis (AE) is caused by the fox tapeworm *Echinococcus multilocularis* and is usually lethal if left untreated. The current strategy for treating human AE is surgical resection of the parasite mass complemented by chemotherapy with benzimidazole compounds. However, reliable chemotherapeutic alternatives have not yet been developed stimulating the research of new treatment strategies such as the use of medicinal plants. The aim of the current study was to investigate the efficacy of the combination albendazole (ABZ) + thymol on mice infected with *E. multilocularis* metacestodes. For this purpose, mice infected with parasite material were treated daily for 20 days with ABZ (5 mg/kg), thymol (40 mg/kg) or ABZ (5 mg/kg) + thymol (40 mg/kg) or left untreated as controls. After mice were euthanized, cysts were removed from the peritoneal cavity and the treatment efficacy was evaluated by the mean cysts weight, viability of protoscoleces and ultrastructural changes of cysts and protoscoleces. The application of thymol or the combination of ABZ + thymol resulted in a significant reduction of the cysts weight compared to untreated mice. We also found that although ABZ and thymol had a scolicidal effect, the combination of the two compounds had a considerably stronger effect showing a reduction in the protoscoleces viability of 62%. These results were also corroborated by optical microscopy, SEM and TEM. Protoscoleces recovered from ABZ or thymol treated mice showed alterations as contraction of the soma region, rostellar disorganization and presence of blebs in the tegument. However both drugs when combined lead to a total loss of the typical morphology of protoscoleces. All cysts removed from control mice appeared intact and no change in ultrastructure was detected. In contrast, cysts developed in mice treated with ABZ revealed changes in the germinal layer as reduction in cell number, while the treatment with thymol or the ABZ + thymol combination predominantly showed presence of cell debris. On the other hand, no differences were found in alkaline phosphatase (AP), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities between control and treated mice, indicating the lack of toxicity of the different drug treatments during the experiment. Because combined ABZ + thymol treatment exhibited higher treatment efficiency compared with the drugs applied separately against murine experimental alveolar echinococcosis, we propose it would be a useful option for the treatment of human AE.

Key words: *Echinococcus multilocularis*, alveolar echinococcosis, albendazole, thymol, liver enzymes

1. INTRODUCTION

Human alveolar echinococcosis (AE) is caused by the fox tapeworm *Echinococcus multilocularis* and is usually lethal if left untreated. Infection of intermediate host such as rodents or accidentally humans is initiated by oral uptake of infectious eggs, which contain the oncosphere larva. After hatching in the host intestine, the oncosphere penetrates the intestinal epithelium and installs in the host organs where develops the metacestode stage. Metacestodes generate almost exclusively in the liver, from where the larva spreads to other organs by infiltration or metastasis (Kern, 2010).

The current strategy for treating human AE is surgical resection of the parasite mass complemented by chemotherapy with benzimidazole compounds (mebendazole or albendazole). For inoperable cases chemotherapy alone is applied. Albendazole (ABZ) inhibits parasite proliferation but it does not cure the disease, meaning patients have to undergo chemotherapy for extended periods of time, resulting in high costs and elevated risk of adverse effects (Torgerson et al., 2008).

Several investigations using *in vivo* rodent models have been carried out looking for alternative treatment for AE. Besides benzimidazoles, these include dicationic diguanidino compounds (Küster et al., 2013); the antimalarials dihydroartemisinin and artesunate (Spicher et al., 2008a) and mefloquine (Küster et al., 2011); the cytostatic drugs vincristine, navelbine and methotrexate (Hübner et al., 2010); 2-methoxyestradiol a compound with documented anti-tumor activity (Spicher et al., 2008b), among others. However, none of the compounds investigated has been translated into clinical application.

Reliable chemotherapeutic alternatives have not yet been developed, stimulating the research of new treatment strategies such as the use of medicinal plants. The pharmaceutical

properties of aromatic plants are partially attributed to essential oils. At present, essential oils are considered as valuable therapeutic options against a number of diseases such as cancer, atherosclerosis, thrombosis, diabetes (Edris, 2006). It has been found that purified compounds derived from essential oils such as carvacrol, eugenol, linalool and thymol inhibit a variety of microorganisms, such as bacteria and fungi (Hulin et al., 1998). Moreover, several essential oils and their constituents have been found to possess antiparasitic activity (Garg, 1997; Hammond et al., 1997).

There are few studies dealing with the role of essential oils specifically against parasitic helminths (Anthony et al., 2005; Hammond et al., 1997; Pessoa et al., 2002). Even though interesting advances have been reported in the in vitro or in vivo application of several essential oils or its components on *E. granulosus* (Maggiore et al., 2012; Albani et al., 2014; Moazeni et al., 2014; Pensel et al., 2014), little is known regarding *E. multilocularis*.

Thymol (2-isopropyl-5-methylphenol) is one of the major components of the essential oils of *Thymus vulgaris* and *Origanum vulgare* and it has been proved to have a strong in vitro and in vivo effect against protoscoleces, microcysts and cysts of *E. granulosus* (Elissondo et al., 2008; 2013; Maggiore et al., 2015). Recently, encouraging findings have been reported using combined drugs ABZ + thymol on *E. multilocularis* protoscoleces and metacestodes *in vitro* (Albani and Elissondo, 2014).

We propose that the simultaneous or sequential application of different drugs is an interesting approach for potentially enhancing effectiveness, shortening long-term use of these substances and therefore decreasing the toxicity. The aim of the current investigation was to investigate the efficacy of the combination ABZ + thymol on mice infected with *E. multilocularis* metacestodes.

2. MATERIALS AND METHODS

2.1. Chemicals

ABZ suspension (0.75 mg/ml) was prepared by dissolution of ABZ pure standard (Sigma-Aldrich), in deionized water (pH=7.0) by shaking on a mechanical shaker (12 h). Thymol (Sigma) was dissolved in olive oil at a drug concentration of 12 mg/ml. ABZ suspension and thymol were vigorously shaken before its intragastric administration to mice.

2.2. Parasite material

All experiments were carried out using parasite isolate 8065 (kindly provided by Klaus Brehm, Institute for Hygiene and Microbiology, University of Würzburg). Cystic masses were dissected from experimentally infected female CF1 mice after 3 month post-infection. Thereafter were pressed through a metal tea strainer and the suspension obtained was washed several times with an antibiotic solution (60 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin in phosphate-buffered saline [PBS]) and maintained in the same solution overnight. Subsequently this preparation was used for mice intraperitoneal inoculation.

2.3. Experimental animals

Animal procedures and management protocols were approved by the Institutional Animal Care and Use Committee (act 2555-07-14) of the Faculty of Exact and Natural Sciences, National University of Mar del Plata, Mar del Plata, Argentina and carried out in accordance with the revised form of The Guide for the Care and Use of Laboratory Animals (National Research Council US, 2011). Unnecessary animal suffering was avoided throughout the study.

Female CF1 mice (body weight 25 g±5) were used. The animals were housed in a temperature-controlled (22 ± 1°C), light-cycled (12 h light/dark cycle) room. Food and water were given *ad libitum.*

2.4. Experimental design

Female CF1 mice (n=40) were infected by intraperitoneal inoculation with 0.5 ml of homogenized metacestode material (8065 strain). At 7 weeks post-infection, mice were allocated into the following experimental groups (10 animals/group) and treated as follows: a) untreated control group, animals receiving 0,3 ml of a mixture of distilled water and olive oil (1:1) as a placebo. b) ABZ group, animals treated with 0.2 ml of ABZ suspension (5 mg/kg); c) thymol group, animal treated with 0.1 ml of thymol (40 mg/kg); d) ABZ + thymol group, animal received a combination of 0.2 ml ABZ suspension (5 mg/kg) and 0.1 ml thymol (40 mg/kg). All treatments were performed by intragastric inoculation every 24 h for 20 consecutive days.

2.5. Determination of efficacy rate of treatments

At the end of the treatment period, the animals were euthanized, and necropsy was carried out immediately thereafter. The cysts were removed from the peritoneal cavity. The treatment efficacy was evaluated by the mean cysts weight, viability of protoscoleces and the ultrastructural study of cysts and protoscoleces.

The weight of the cysts collected from each individual animal was recorded using an analytical balance. The efficacy of treatments (based on the weight of cysts from infected mice), was calculated using the following formula: the mean weight of untreated control group minus the mean cysts weight of treated group divided by the mean cysts weight of untreated group.

Cystic masses from each individual (treated and untreated animals) were pressed through a metal tea strainer and the suspension obtained was washed several times with PBS. Then the suspension was vigorously shaken for approximately 10 min to release the protoscoleces from the metacestode material. Afterwards, the suspension was sequentially sieved through a polyester gauze 150 μm pore size and then through a 30 μm pore size (Gasatex, Argentina). Protoscoleces were retained on the top of the 30 μm pore size gauze and collected. Protoscoleces vitality was

performed using the methylene blue exclusion test (Casado et al., 1986). Dead protoscoleces stain blue and those that are alive exclude the dye and remain clear.

2.6. Morphologic study

Samples of protoscoleces collected from treated and untreated mice were stained with methylene blue and observed under light microscopy to asses morphological differences. Samples of protoscoleces and cysts collected from treated and untreated mice were processed for scanning and transmission electron microscopy (SEM and TEM) as described by Elissondo et al. (2006, 2007).

2.7. Enzyme assays

Alkaline phosphatase (AP), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities were measured in plasma of control and treated mice.

Blood samples collected in 500 I.U. per ml heparin were obtained from animals immediately before the euthanasia. Plasma was separated by centrifugation at 2,000 ×g for 15 min, placed into plastic tubes and frozen at -20°C until analysis.

Enzyme activities were determined from heparinized plasma following the procedure described below. In all cases, the activities were measured at 37 °C in a automatic analyzer Metrolab Wiener®, the volume of the reaction mixture was 110 µl with 100 µl of enzyme samples and measurements made after 180 sec of incubation. The results were expressed Units/Litre (U/L).

Alkaline phosphatase (AP) activity was measured based on (Bessey et al., 1946).

For estimation of GOT and GPT the optimized UV method (IFCC) was followed (Bergmeyer et al., 1976).

2.8. Statistical analysis

Data are reported as arithmetic mean (±SD) and statistical analysis was performed using the Instat 3.0 Software (GraphPad Software, CA, USA). Cysts weights, protoscoleces viability and enzyme data were compared by means of Kruskal–Wallis (non parametric ANOVA) followed by Dunn's multiple comparison test. A value of $P<0.05$ was considered statistically significant.

3. RESULTS

Hydatid cysts developed in all the infected animals involved in the efficacy studies. No significant difference was found in mean cyst weight between ABZ and control group ($P> 0.05$) although there was a reduction of approximately 48% in the cyst weight (4.69 ± 1.48 g versus 9.07 ± 2.46 g in controls). The combination of ABZ + thymol resulted in a higher reduction of the parasite weights (1.5 ± 1.4 g) and this was highly significant ($P<0.001$). Thymol alone caused a reduction of cyst weight of approximately 60% (Table 1).

Cysts isolated from mice treated with the combination ABZ + thymol lacked the typical structure when observed macroscopically. They were collapsed and the cyst wall was always thinner (data not shown).

Following the isolation of protoscoleces from control hydatid cysts, the methylene blue viability test revealed that > 99 % of the recovered untreated parasites were still viable. The viability of *E. multilocularis* protoscoleces isolated from treated mice is shown in Fig. 1. There was no statistically significant difference between the viability of protoscoleces recovered from control and ABZ-treated groups. Although thymol and ABZ + thymol treatments had a scolicidal effect, the combination of the two compounds caused a higher reduction in the protoscoleces viability (Fig. 1).

Protoscoleces isolated from control mice showed typical morphology (Fig. 2A) while protoscoleces recovered from treated mice lacked normal structure showing soma contraction, rostellar disorganization and presence of blebs in the tegument (Fig. 2B-D). These results were also sustained by SEM (Fig. 3). ABZ or thymol alone caused the lost of the normal morphology of protoscoleces including soma contraction, rostellar disorganization, disruption of microtriches pattern and partial loss of hooks (Fig 3B-C). Interestingly, both drugs combined in all the cases lead to a total loss of the typical morphology of protoscoleces (Fig. 3D).

Metacestode tissues obtained from treated and control mice were inspected by SEM (Fig. 4) and TEM (Fig. 5). All cysts in the samples removed from control mice appeared intact and no change in ultrastructure were detected (Fig. 4A). Moreover different steps in the protoscoleces formation could be observed (Fig. 4B) confirming the proliferative activity of the cyst. In contrast, the ultrastructural study of cysts developed in mice treated with ABZ (Fig. 4D) revealed changes in the germinal layer as reduction in cell number, while the treatment with thymol (Fig. 4C) or the ABZ + thymol combination (Fig. 4E) predominantly showed presence of cell debris.

TEM analysis of cysts recovered from the untreated control group revealed an intact germinal layer composed of numerous cell types (Fig. 5A). Tegument structure was normal with numerous microtriches protruding into the laminated layer. Parasites recovered from thymol (Fig. 5B) and ABZ-treated mice (Fig. 5C) exhibited modifications such as distorted internal tissue with presence of vacuolated areas. On the other hand, metacestodes obtained from ABZ + thymol treated animals (Fig. 5D) were damaged showing microtriches largely distorted or no longer present.

No statistical differences ($P>0.05$) were found in AP, GOT and GTP activities between control and treated mice (Fig. S1).

4. DISCUSSION

Human AE, caused by the larval stage of the fox tapeworm *E. multilocularis* is amongst the world's most dangerous zoonoses (Torgerson et al., 2010). Unfortunately, a large number of cases do not respond favourably to chemotherapy with benzimidazoles and these drugs produce stabilization, rather than cure in the majority of patients. With regard to these difficulties, novel therapeutical tools are needed to optimize treatment of cystic echinococcosis.

Earlier in vitro and in vivo investigations had already demonstrated the effectiveness of thymol against *E. granulosus* protoscoleces and hydatid cysts (Elissondo et al., 2008; 2013; Maggiore et al., 2015), *Mesocestoides corti* tetrathyridia and adult worms (Maggiore et al., 2014), and also promising *in vitro* results were obtained using the combination ABZ + thymol on *E. multilocularis* protoscoleces (Albani and Elissondo, 2014).

In this study, the combination of ABZ + thymol was evaluated on experimentally infected mice with *E. mutilocularis* and, the effects were directly compared to uninfected mice or mice treated with a single drug.

Hydatid cysts developed in all the infected animals involved in the clinical efficacy study. The treatment with thymol or the combination ABZ + thymol lead to a significantly reduction in cyst weights compared to those recovered from untreated mice. Even though the treatment with ABZ did not result in a statistically significant reduction in cyst weight, evident damage could be detected both in protoscoleces and germinal layer of cysts.

No ultrastructural changes were observed either in the germinal layer of cysts or protoscoleces recovered from untreated mice. In contrast, the germinal layer of cysts isolated from ABZ or thymol treated mice was markedly altered. Nevertheless, the combination ABZ + thymol caused the highest effect both on protoscoleces as on germinal layer of cysts suggesting a

synergistic effect of the two drugs. We found a positive correlation between the level of damage of treated cysts and the protoscoleces vitality and its morphological changes.

From the analysis of thymol chemical structure, this compound would have an amphipathic and/or hydrophobic behavior. This suggests the ability of thymol to partition in the membrane from an aqueous phase as well as a capacity to affect the membrane organization and the electrostatic surface (Elissondo et al., 2008).

The in vivo cytotoxic activity of thymol was evaluated by Robledo et al., (2005). At an orally dosage of 40 mg/kg of body weight/day, thymol was not toxic to Golden hamsters based on corporal weight, behaviour and serum levels of bilirubin, uric acid and glucose. Moreover, the in vivo effect of thymol on the *E. granulosus* murine model was observed by us (manuscript on revision). The chemoprophylactic activity and the effects on secondary hydatid disease of thymol were demonstrated working with a dose of 40 mg/kg of body weight/day. No toxic effects were detected on CF1 mice during the time that lasted the experiment. In our experiments on murine alveolar echinococcosis, this lack of toxicity was also evident since none of the mice exhibited adverse effects during the entire treatment period and the liver enzymes values were similar to the profiles of control mice. However, to discard possible chronic toxicity of thymol, we suggest that long term evaluation of genotoxicity or mutagenicity indicators should be performed.

Even though lesions from infected human patients rarely exhibit brood capsule and protoscolex formation within vesicles, some exceptions have been reported (Gottstein et al., 2014). The *E. multilocularis* isolate used in these experiments developed numerous protoscoleces in the murine model. It allowed us to asses not only the drug effects on the cysts but also on the protoscoleces. We propose that results obtained with the protoscoleces treatment could be extrapolated to other parasitic tapeworms as *E. granulosus* in which protoscoleces formation is

usual. However should be considered that the structure of the *E. granulosus* cyst is very different from *E. multilocularis* which could interfere with the drug activity.

In summary, combined ABZ + thymol treatment exhibited higher efficacy compared with the drugs applied separately against murine experimental AE. In future, it would be interesting to adjust the dose and duration of treatment in order to further enhance the efficacy of the combination of both drugs. Finally, we propose it would be a useful option for the treatment of human AE.

5. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

6. ACKNOWLEDGMENTS

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Figure caption

Table 1. Mean (±SD) weights (g) of the parasite recovered from infected mice treated with albendazole (ABZ) suspension (5 mg/kg), thymol (40 mg/kg) or ABZ (5 mg/kg) + thymol (40

mg/kg) combination therapy. Mice after 7 weeks of parasite inoculation were treated during 20 days, every 24 h. * *P<0.001* statistically significant differences between ABZ + thymol group vs control group. ** *P<0.01* statistically significant differences between thymol group vs control group. *** *P<0.05* statistically significant differences between treated ABZ + thymol vs ABZ suspension group.

Figure 1. Survival of *Echinococcus multilocularis* protoscoleces recovered from mice treated with albendazole (ABZ), thymol and the combination of both drugs. Each point represents the mean percentage of viable protoscoleces from ten different individuals ± SD.

Figure 2. Light microscopy of *Echinococcus multilocularis* protoscoleces isolated from: A. untreated mice, note the typical morphology of protoscoleces with presence of abundant calcareous corpuscles (450x). B. Mice treated with thymol at a dose of 40 mg/kg during 20 days (400x). C. Mice treated with albendazole (ABZ) at a dose of 5 mg/kg during 20 days (600x). D. Mice treated with the combination of ABZ (5 mg/kg) + thymol (40 mg/kg) during 20 days (400x). Alterations such as soma contraction (white arrows), rostelar disorganization (arrow heads) and presence of blebs in the tegument (black arrows) can be observed.

Figure 3. Scanning electron microscopy of *Echinococcus multilocularis* protoscoleces isolated from: A. untreated mice. B. Mice treated with thymol at a dose of 40 mg/kg during 20 days. Note soma contraction (arrows). C. Mice treated with albendazole (ABZ) at a dose of 5 mg/kg during 20 days. Soma contraction and rostelar disorganization can be noticed. D. Mice treated with ABZ (5 mg/kg) + thymol (40 mg/kg) during 20 days. Total loos of the typical morphology of protoscoleces can be observed. Scale bar: A-D 20 µm.

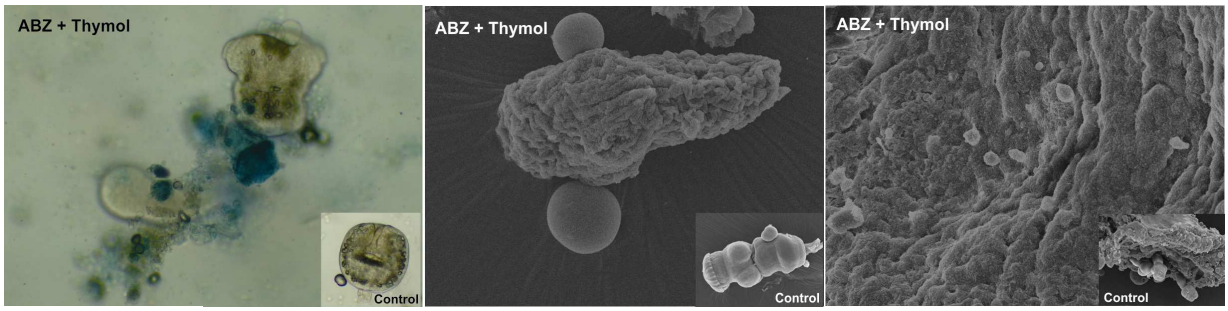
Figure 4. Scanning electron microscopy of *Echinococcus multilocularis* metacestodes recovered from: A. Untreated mice. B. High magnification image showing nascent protoscoleces. C. Mice treated with thymol at a dose of 40 mg/kg during 20 days. The germinal layer (gl) is altered. D. Mice treated with albendazole (ABZ) at a dose of 5 mg/kg during 20 days. Note the reduction in cell number compared to control. E. Mice treated with ABZ (5 mg/kg) + thymol (40 mg/kg) during 20 days. Alterations in the germinal layer (gl) and only cell debris can be observed. Scale bar: A 50 µm, B 100 µm, C 20 µm, D 50 µm, E 20 µm.ll: laminated layer; gl germinal layer.

Figure 5. Transmission electron microscopy of *Echinococcus multilocularis* metacestodes recovered from: A. Untreated mice. The intact laminated layer (LL), germinal layer (GL) and tegument (Teg) are distinguishable. Arrows point towards microtriches that protrude into the laminated layer (10,000x). B. Mice treated with thymol at a dose of 40 mg/kg during 20 days. Note that microtriches are greatly reduced in length and number (25,000x). C. Mice treated with albendazole (ABZ) at a dose of 5 mg/kg during 20 days. Distorted internal tissue with presence of vacuolated areas can be noticed (15,000x). D. Mice treated with ABZ (5 mg/kg) + thymol (40 mg/kg) during 20 days. The germinal is altered. Note microtriches largely distorted or no longer present (15,000x).

Figure S1. Alkaline phosphatase (AP), glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities in plasma of control and mice treated with albendazole (ABZ), thymol or the combination ABZ + thymol. There was no statistical differences between control and the different treatments in any of the studied enzymes ($P>0.05$).

Table I.

	Clinical efficacy study	
	Wet weight (g) of cysts Mean±SD	**% of efficacy**
Unmedicated control group	9.07±2.46	
ABZ suspension	4.69±1.48	48.29
Thymol	3.62±1.25**	60.08
ABZ + thymol	1.5±1.4* ***	83.46



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