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

Clara María Albani[a,b], Patricia Eugenia Pensel[a,b], Natalia Elissondo[c], Guillermo Gambino[c], María Celina Elissondo[a,b,*]

[a] *Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, 7600 Mar del Plata, Argentina*
[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*
[c] *Laboratorio de Análisis Clínicos Santisteban, 7000 Tandil, Buenos Aires, Argentina*

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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.

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.

* Corresponding author at: Laboratorio de Zoonosis Parasitarias, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata (UNMdP), Funes 3350, 7600 Mar del Plata, Argentina.
E-mail addresses: mceliss@mdp.edu.ar, c.elissondo@gmail.com (M.C. Elissondo).

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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; Hemphill et al., 2014).

Several investigations using in vivo rodent models have been carried out looking for alternative treatment for AE. Besides benzimidazoles, these include nitazoxanide (Stettler et al., 2004); amphotericin B (Reuter et al., 2010); 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), amongst 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 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