Parasitology International xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Chemoprophylactic and therapeutic efficacy of thymol in murine cystic echinococcosis

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ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form 15 June 2015
Accepted 17 June 2015
Available online xxxx

Keywords:
Cystic echinococcosis
Echinococcus granulosus
Essential oils
Thymol
Albendazole

ABSTRACT

Cystic echinococcosis is a zoonotic disease caused by the larval stage of the cestode *Echinococcus granulosus*. The drugs commonly used against cystic echinococcosis are benzimidazoles. Unfortunately, 20%–40% of cases do not respond favorably to such chemotherapy. Consequently, the search of new therapeutic alternatives such as the use of traditional medicinal plants has been increased. The aim of the current experimental work was to investigate the chemoprophylactic and clinical efficacy of thymol on mice infected with *E. granulosus* metacestodes. Thymol (40 mg/kg) was administered under two different therapeutic schemes: dosing every 24 h over 20 days and treatment every 12 h for 10 days. Thymol demonstrated efficacy against experimental murine cystic echinococcosis. The chemoprophylactic and therapeutic effect of thymol was comparable to that of albendazole. Due to the lack of toxicity observed in mice at the tested doses; we consider that thymol is a potential alternative to be applied for the treatment of human hydatid disease.

1. Introduction

Cystic echinococcosis, an infection with the larval form of the dog tapeworm *Echinococcus granulosus*, is characterized by cystic lesions, most commonly in the liver and lungs [1]. This parasitic infection is a chronic, complex, and still neglected disease [2].

Currently four treatment approaches are in use: surgery, PAIR (puncture, aspiration, injection of protoscolicidal agent, reaspiration), chemotherapy, and watch and wait for inactive, clinically silent cysts [3]. The drugs commonly used against cystic echinococcosis are benzimidazoles, such as albendazole (ABZ) and mebendazole [4]. Unfortunately, 20%–40% of cases do not respond favorably to such chemotherapy and these drugs produce stabilization, rather than cure in the majority of patients [5]. With regard to these difficulties, novel therapeutical tools are needed to optimize treatment of cystic echinococcosis.

The control of helminthosis, and generally of all parasitic diseases, is usually made with synthetic anthelmintics. These anthelmintic drugs have some important disadvantages, such as cost and improper use leading to drug resistance, environmental pollution, and food residues [6]. Consequently, the search of new therapeutic alternatives such as the use of traditional medicinal plants has been increased.

Aromatic plants have pharmaceutical properties that are in part attributed to essential oils [7]. For their hydrophobic character, the essential oils and their components have a considerable potential of pharmacological applications as antimicrobial agents [7,8]. The role of essential oils against parasitic helminths has been studied by several authors [9–12]. However, there are few publications about the effect of these substances on *E. granulosus*. The in vitro effect of the essential oils of *Rosmarinus officinalis* (rosemary), *Mentha pulegium*, *M. piperita*, *Pistacia khinjuk* (pistachio) and *Trachyspermum ammi* (ajowan) has been demonstrated against protoscoleces of *E. granulosus* [13–16].

Thymol (2-isopropyl-5-methylphenol) is one of the major components of the essential oils of *Thymus vulgaris* and *Origanum vulgare* and is a widely known anti-microbial agent [17]. The in vitro and in vivo activities of thymol against *Leishmania panamensis* [18] and *Anisakis simplex* larvae [19] were proven. Moreover, several encouraging findings have been reported using thymol in vitro on *E. granulosus* protoscoleces, microcysts and metacestodes [20,21]. Thymol had anthelmintic effect against the larval stage, altering the viability, structure and ultrastructure of the cestode.

The aim of the current experimental work was to investigate the chemoprophylactic and clinical efficacy of thymol on mice infected with *E. granulosus* metacestodes.

2. Materials and methods

2.1. Chemicals

ABZ suspension (2.1 mg/ml) was prepared by dissolution of ABZ pure standard (Pharmaceutical grade, Parafarm, Buenos Aires, Argentina) in

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http://dx.doi.org/10.1016/j.parint.2015.06.005
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deionized water (pH = 7.0) under shaking (12 h). ABZ suspension was vigorously shaken before its intragastric administration to mice.

Thymol was obtained from Sigma-Aldrich (USA). Thymol solution (2.4 mg/ml) was prepared by dissolution of the drug in olive oil and maintained under refrigeration (3 to 5 °C).

2.2. Protoscolex collection

Protoscoleces of *E. granulosus* were collected aseptically from liver and lung hydatid cysts of infected cattle slaughtered in an abattoir located in the southeast of Buenos Aires province, Argentina. The area where the cattle came from is known to include only the G1 strain of *E. granulosus* [22]. Viability was assessed as previously described [23].

2.3. Experimental animals and infection

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 2011 revised form of *The Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. Unnecessary animal suffering was avoided throughout the study. Female CF-1 mice (n = 100; body weight 25 g ± 5) were infected by intraperitoneal inoculation with 1500 *E. granulosus* protoscoleces/animal, suspended in 0.5 ml of medium 199 (Gibco). The animals were housed in a temperature controlled (22 ± 1 °C), light-cycled (12-h light/dark 174 cycle) room. Food and water were given ad libitum.

2.4. Experimental design

Two different experimental designs were conducted: a chemoprophylactic efficacy study (which simulates a cyst rupture during surgical practice and the concomitant drug treatment) and, a clinical efficacy study (simulating an experimental secondary hydatidosis).

2.4.1. Chemoprophylactic efficacy study

At the time point of infection, the animals were allocated into 5 experimental groups (n = 10) and treated as follows: a) distilled water control group, animals receiving distilled water as a placebo; b) oil control group, animals receiving olive oil as a placebo; c) ABZ group, animals were treated with ABZ suspension at the dose rates of 25 mg/kg every 24 h for 30 days; d) thymol group I, animals were treated with thymol at the dose rates of 40 mg/kg every 24 h for 20 days; and e) thymol group II, animals were treated with thymol (40 mg/kg) at 12-h intervals over 10 days. Treatments were performed by intragastric administration. Six months after infection, mice were euthanized, and necropsy was carried out immediately thereafter.

2.4.2. Clinical efficacy study

Protoscoleces of *E. granulosus* can differentiate to hydatid cysts if released within (or passaged into) an intermediate host. Cystic differentiation is completed after 2–3 months [24]. At 6 months post-infection, mice were allocated into the following experimental groups (10 animals/group) and treated as follows: a) distilled water control group, animals receiving distilled water as a placebo; b) oil control group, animals receiving olive oil as a placebo; c) ABZ group, animals were treated with ABZ suspension at the dose rates of 25 mg/kg every 24 h for 30 days; d) thymol group I, animals were treated with thymol at the dose rates of 40 mg/kg every 24 h for 20 days; and e) thymol group II, animals were treated with thymol (40 mg/kg) at 12-h intervals over 10 days. Treatments were performed by intragastric administration. At the end of the treatment period, animals were euthanized, and necropsy was carried out immediately thereafter.

2.5. Determination of parasite weight and morphologic study

At necropsy in both the prophylactic and efficacy studies, the peritoneal cavity was opened, and the hydatid cysts were carefully removed. The weight of the cysts collected from each individual animal was recorded using an analytical scale. Samples of cysts recovered from each mouse were processed for scanning and transmission electron microscopy (SEM and TEM) as described by Elissondo et al. [25].

2.6. Statistical analysis

Weight of cysts (reported as mean ± SD) 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. The statistical analysis was performed using the Instat 3.0 software program (GraphPad Software, San Diego, CA).

3. Results

3.1. Chemoprophylactic efficacy study

All of the infected mice (10/10) from the untreated control groups, ABZ-treated group and thymol-treated group I developed metacestodes in the abdominal cavity. On the contrary, in 1 out of the 10 thymol-treated group II mice, the infection did not progress. Statistically significant differences (ABZ group: $P < 0.001$; thymol group I: $P < 0.01$; thymol group II: $P < 0.05$) were observed in the weights of cysts recovered from untreated mice compared with those from the treated groups. On the other hand, no statistically significant differences were found between the weights of cysts recovered from the two thymol-treated groups and ABZ-treated group ($P > 0.05$) (Table 1).

All cysts removed from control mice appeared turgid, showing no observable collapse of the germinal layer, and no changes in ultrastructure were detected by SEM (Fig. 1a). TEM analysis of cysts recovered from the untreated control group revealed the typical features of *E. granulosus* metacestodes, with a distinct acellular outer laminated layer and a germinal layer without alterations (Fig. 1b). In contrast, the ultrastructural study of cysts developed in mice treated with ABZ and thymol revealed a normal laminated layer, but some alterations on the germinal layer were detected (Fig. 2). Studies by SEM revealed that the germinal layer of treated cysts lost the feature multicellular structure (Fig. 2a, c and e). TEM analysis of cysts from treated mice revealed the presence of some signs of degeneration as an increment in the number of vacuoles and lipid droplets (Fig. 2b, d and f).

3.2. Clinical efficacy study

Table 2 summarizes the cyst weights (mean ± SD) recorded after treatments on the different experimental groups (unmedicated control and treated groups) involved in the clinical efficacy study. Both thymol

Table 1
Chemoprophylactic efficacy study. Mean (±SD) weights (g) of hydatid cysts recovered at six months post-infection from artificially infected mice from the unmedicated control and treated groups. Treatments were given at 40 mg/kg, every 24 h over 20 days or every 12 h over 10 days.

	Chemoprophylactic efficacy study
	Wet weight (g) of cysts Mean ± SD
Unmedicated control group (olive oil)	12.3 ± 4.2
Unmedicated control group (distilled water)	10.6 ± 2.9
ABZ suspension	1.3 ± 0.9***
Thymol group I	2.9 ± 2.3**
Thymol group II	3.7 ± 2*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; statistically significant differences between treated group vs control group.

Please cite this article as: M. Maggiore, et al., Chemoprophylactic and therapeutic efficacy of thymol in murine cystic echinococcosis, Parasitology International (2015), http://dx.doi.org/10.1016/j.parint.2015.06.005