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Research paper

Experimental cystic echinococcosis therapy: In vitro and in vivo combined 5-fluorouracil/albendazole treatment

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ABSTRACT

Human cystic echinococcosis is a zoonosis caused by the larval stage of the tapeworm *Echinococcus granulosus* sensu lato. Although benzimidazole compounds such as albendazole (ABZ) and mebendazole have been the cornerstone of chemotherapy for the disease, there is often no complete recovery after treatment. Hence, new strategies are required to improve treatment of human cystic echinococcosis. The goals of the current study were as follows: (i) to evaluate the in vitro efficacy of the 5-fluorouracil (5-FU) and ABZ combination against *E. granulosus* sensu lato (s. l.) protoscoleces and cysts, (ii) to compare the clinical efficacy of 5-FU alone or in combination with ABZ in infected mice. The combination of 5-FU + ABZ had a stronger in vitro effect against larval stage than that did both drugs alone. Even at the lowest concentration of 5-FU + ABZ combination (1 μg/ml), the reduction of the viability of protoscoleces and cysts was greater than that observed with drugs alone at 10 μg/ml. The results were confirmed at the ultrastructural level by scanning electron microscopy. These data helped to justify the in vivo investigations assessing the therapeutic potential of the combination of 5-FU and ABZ suspension in CF-1 mice infected with *E. granulosus* s. s. metacestodes. Treatment with 5-FU (10 mg/kg) or 5-FU (10 mg/kg) + ABZ suspension (5 mg/kg) reduced the weight of cysts recovered from mice compared with control groups. Interestingly, the effect of 5-FU given weekly for 5 consecutive weeks was comparable to that observed with ABZ suspension under a daily schedule during 30 days. Co-administration of 5-FU with ABZ did not enhance the in vivo efficacy of drugs alone calculated in relation to cysts weights. However, the combination provoked greater ultrastructural alterations compared to the monotherapy. In conclusion, we demonstrated the efficacy of 5-FU either alone or co-administrated with ABZ against murine experimental cystic echinococcosis. Since 5-FU treatments did not cause toxic effect in mice, further in vivo studies will be performed by adjusting the dosage and the frequency of treatments.

1. Introduction

Cystic echinococcosis (CE), a zoonosis caused by the larval stage of *Echinococcus granulosus* sensu lato (s. l.), is characterized by long term growth of hydatid cysts in humans and mammalian intermediate hosts (McManus et al., 2012). This parasitic infection is a chronic, complex, and still neglected disease (Brunetti et al., 2011).

The WHO-IWGE classification provides the basis for choosing basically four treatment and management options for CE: surgery, percutaneous sterilization, chemotherapy with benzimidazoles (BZ) and observation (watch and wait) for inactive, clinically silent cysts (Brunetti et al., 2011). Each of these therapeutic tools has limitations depending on the individual case. The evidence supporting any of these modalities from carefully designed clinical studies is insufficient and the choice of treatment options remains controversial (Stojkovic et al., 2009).

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Medical treatment is indicated when surgical removal is not appropriate for patients with multiple cysts in two or more organs, for prevention of secondary echinococcosis after surgery and in some cases for presurgical treatment (Pawlowski et al., 2001). The drugs commonly used for anti-hydatid cysts treatment are BZ, such as albendazole (ABZ) and mebendazole (MBZ) (McManus et al., 2012). The pharmacological activity mediating BZ action is based on binding to parasite β-tubulin, which produces a subsequent disruption of the tubulin-microtubule dynamic equilibrium (Lacey, 1990). Albendazole and MBZ are given at the WHO recommended dosages of 10–15 mg/kg/day and 40–50 mg/kg/day, respectively (WHO-IWGE, 2001).

The response to BZ treatment is frequently unpredictable (El-On, 2003). Approximately a third of patients treated with BZ drugs have been cured, 30–50% develop some evidence of a therapeutic response while between 20 and 40% of cases do not respond favorably (Moro and Schantz, 2009). Moreover, BZ sometimes acts exclusively as a parasitostatic agent, and relapses after chemotherapy have been reported (Stamatakos et al., 2009). The large variability observed in the therapeutic success of BZ anthelmintics on CE may be explained by the host immunological status and/or the features of the cysts, including their size and location. Furthermore, the poor/erratic gastrointestinal absorption is a common inconvenience for the systemic availability of orally administered BZ in most species (Lanusse and Prichard, 1993). Hence, novel and improved therapeutic tools are needed in order to optimize treatment of CE.

De novo drug discovery offers big challenges in scientific and financial investment, often with low returns in terms of the number of drugs that finally make it to the market (Nosengo, 2016). This problem is further amplified in neglected tropical disease such as CE, where existing resources are strained and the financial returns low. Therefore, novel chemotherapeutics for echinococcosis have to be identified issuing from existing drugs by one of the following strategies: (1) in vitro testing of broad-spectrum anti-infective drugs, either in parallel with, or followed by, small animal experimentation; (2) in vitro testing of drugs inhibiting proliferation of cancer cells for their effects on the viability of *Echinococcus* metacestodes and protoscoleces (Hemphill and Müller, 2009).

There are a number of similarities between cancer cells and some parasites (Klinkert and Heussler, 2006). Particularly, *Echinococcus* spp. metacestodes exhibit tumor-like properties, as reflected by their seemingly unlimited growth and proliferation potential, and their abilities to modulate the immune response and to form metastases (Hemphill and Müller, 2009). Consequently, the in vitro or/and in vivo effect of several antitumor drugs including genistein and the genistein derivative Rm6423 (Naguleswaran et al., 2006), 2-methoxyestradiol (Spicher et al., 2008a), artemisinin and artemisinin derivatives (Spicher et al., 2008b), tamoxifen (Nicolao et al., 2014), paclitaxel (Pensel et al., 2014), osthole (Yuan et al., 2016) and carbazole aminoalcohols (Wang et al., 2017) has been evaluated against *Echinococcus granulosus* s. l. larval stage.

The fluoropyrimidine, 5-fluorouracil (5-FU), is an antimetabolite drug that is widely used in the treatment of a range of cancers including breast and aerodigestive tract, but it has the greatest impact in colorectal tumors. 5-Fluorouracil exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA (Longley et al., 2003). In previous works, we reported the in vitro effect of 5-FU on the larval stage of *E. granulosus* s. l. At clinically achievable concentrations, 5-FU severely inhibits the survival of the germinal cells, protoscoleces and cysts (Pensel et al., 2014). Therefore, in the search of new strategies for CE treatment, it could be interesting to evaluate the effect of 5-FU alone or in combination with ABZ on the murine model of cystic echinococcosis.

The goals of the current study were as follows: (i) to evaluate the in vitro efficacy of the 5-FU and ABZ combination against *E. granulosus* s. l. protoscoleces and cysts, (ii) to compare the clinical efficacy of 5-FU alone or in combination with ABZ in infected mice.

2. Materials and methods

2.1. Chemicals

For in vitro studies, the stock solutions of ABZ (Parafarm, Buenos Aires, Argentina) and 5-FU (Roche Laboratories, Neuilly-sur-Seine, France) were prepared by dissolution in dimethyl sulphoxide (DMSO) at a drug concentration of 10 mg/ml.

For in vivo studies, ABZ suspension (0.75 mg/ml) was prepared by dissolution of ABZ in deionized water (pH = 7.0) under shaking (12 h). 5-FU solution (4.5 mg/ml) was prepared by dissolution of 5-FU in sterile saline under shaking (12 h).

2.2. Ethic statement and experimental animals

Animal procedures and management protocols were approved by the Institutional Animal Care and Use Committee (RD 148/15) of the Faculty of Exact and Natural Sciences, National University of 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 CF-1 mice (n = 55, body weight 25g ± 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.

2.3. Protoscoleces and cysts collection

Protoscoleces 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. Viability was assessed by the methylene blue exclusion test (Elissondo et al., 2006). *E. granulosus* genotype was determined by sequencing a fragment of the gene coding for mitochondrial cytochrome c oxidase subunit 1 (CO1), as previously described (Cucher et al., 2011). Based on sequencing analysis, the G1 genotype (sheep strain) was identified.

Murine cysts were obtained from the peritoneal cavities of mice (n = 5) infected with *E. granulosus* s.s. protoscoleces as was described by Fabbri et al. (2016).

2.4. In vitro drug activity study on protoscoleces and cysts

Both protoscoleces (1500) and cysts (groups of 10 cysts with 2–5 mm of diameter) were cultured in 10 ml of medium 199 (Lab. Microvet SA, Argentina), supplemented with antibiotics and glucose as previously described (Fabbri et al., 2016).

The stock solutions of ABZ and 5-FU were added to the medium 199 either separately or in combination at the following final concentrations: 10 μg/ml ABZ, 5 μg/ml ABZ, 1 μg/ml ABZ, 10 μg/ml 5-FU, 5 μg/ml 5-FU, 1 μg/ml 5-FU, 10 μg/ml ABZ + 10 μg/ml 5-FU, 5 μg/ml ABZ + 5 μg/ml 5-FU and 1 μg/ml ABZ + 1 μg/ml 5-FU. The biggest volume of drug solution added to the medium was 0.2%. Protoscoleces and cysts incubated in culture medium with 0.2% DMSO serve as control. All experiments were performed in triplicate and were repeated three times.

2.4.1. Protoscoleces

Culture tubes were followed microscopically every day to determine the appearance of morphological alterations. Samples of protoscoleces from each group were taken for viability assessment using the methyl-

ene blue exclusion test. Additionally, ultrastructure studies with scanning electron microscope (SEM) were performed (Fabbri et al., 2016).

2.4.2. Cysts

Culture tubes were followed macro-and microscopically every day. Samples of cysts from each group were taken and then fixed for SEM. The criteria for cyst viability assessment included the loss of turgidity, the collapse of cysts, and the ultrastructural observation of the germinal layer (Fabbri et al., 2016).

2.5. Clinical efficacy study in mice infected with E. granulosus s. s.

Female CF-1 mice (n = 50) were infected by intraperitoneal inoculation with *E. granulosus* s. s. protoscoleces following the procedures described in Fabbri et al., 2016. At 5 months post-infection, the animals were allocated into the following experimental groups (10 animals/group): (1) Unmedicated control group, animals receiving 0.3 ml deionized water as a placebo by oral route; (2) Saline control group, animals were administrated with 0.1 ml physiological solution by intravenous (i.v.) injection into the lateral tail vein; (3) ABZ-SUSP group, animals were treated with 0.3 ml ABZ suspension by oral route; (4) 5-FU group, animals were treated with 0.1 ml 5-FU by the i.v. route; (5) ABZ-SUSP + 5-FU group, animal received a combination of 0.3 ml ABZ suspension by oral route and 0.1 ml 5-FU by i.v. injection.

Treatment with ABZ suspension was performed daily during 30 days at the dose of 5 mg/kg 5-Fluorouracilo was given once per week during 30 days at the dose of 10 mg/kg. The treatment protocol of the five experimental groups of mice is shown in Fig. 1. To avoid a possible circadian variation in the activities of the major enzymes involved in 5-FU metabolism (orotate phosphoribosyltransferase and dihydrouracil dehydrogenase), all mice were treated at the same time starting at 1:00 P.M. (el Kouni et al., 1990; Naguib et al., 1993).

2.6. Animal health status assessment

Animals were observed daily for signs of morbidity and these data were recorded on a comprehensive health monitoring standard operating procedure which included assessments of body weight loss, change in appetite and in stool consistency as well as behavioral and appearance alterations.

The average body weight change (BWC) in all groups was calculated using the formula:

$$BWC = (ABW_n/ABW_1) \times 100 - 100\%$$

where ABW_n is the average body weight on the nth-day of experiment (during treatment) and ABW_1 is the average body weight on the first day of treatment. Mice were euthanized if body weight loss (BWL) exceeded 20%, or if significant deteriorations were observed in mouse health (Hare et al., 2013).

2.7. Blood biochemical assay

At the end of treatment period, animals were anesthetized with a mixture of ketamine (100 mg/kg)/xylazine (10 mg/kg) and blood samples were collected. Plasma was separated by centrifugation at 2000 × *g* for 15 min, placed into plastic tubes and frozen at −20 °C until analysis. In the present study, liver function was evaluated with serum levels of alkaline phosphatase (ALP), glutamate pyruvate transaminase (GPT) and gamma-glutamyl transpeptidase (GGT). Nephrotoxicity was determined by blood urea and creatinine (Cr).

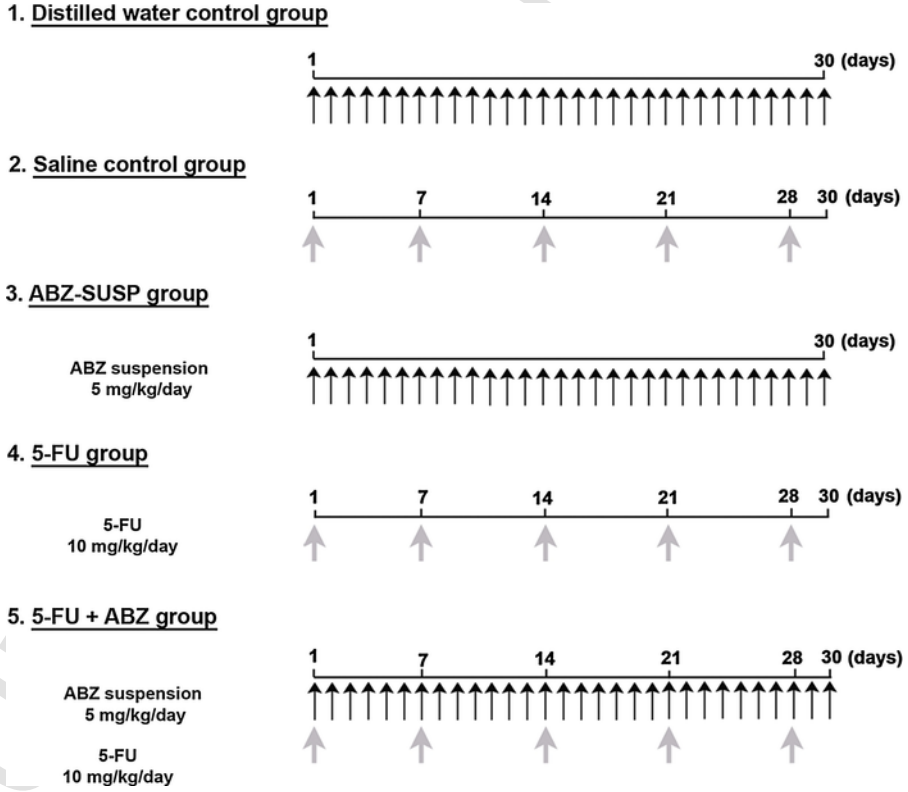

Fig. 1. Experimental protocol for the in vivo efficacy of ABZ and 5-FU alone or combined with ABZ in mice infected with *E. granulosus* s. s. metacestodes. The groups were as follows: 1) control group, treated with deionized water daily by oral route during 30 days; 2) saline control group, treated with sterile saline by i.v. injection, once a week during 30 days; 3) ABZ-SUSP group, treated with ABZ suspension by oral route daily during 30 days; 4) 5-FU group, treated with 5-FU by i.v injection, once a week during 30 days; 5) 5-FU + ABZ-SUSP group, 5-FU injected once a week and ABZ suspension administrated orally for 30 days. At the end of treatment period, animals were euthanized, and necropsy was carried out immediately thereafter. The cysts were removed from the peritoneal cavity.

These parameters were all measured in plasma samples using commercial kits from Wiener Lab (Rosario, Argentina).

The activity of ALP was measured using protocols described by Bessey et al. (1946). For estimation GPT the optimized UV method (IFCC) was followed (Bergmeyer et al., 1976). Plasma GGT activity was determined using a modified Szasz method (IFCC) (Szasz, 1969; Shaw et al., 1983). The enzyme activities were expressed as Units/Litre (U/L).

Uremia was determined by a specific enzymatic method for blood urea (Talke and Schubert, 1965). For the quantification of Cr in plasma, a kinetic method (Jaffe reaction) was used according to Owen et al. (1954). Urea and Cr concentrations were expressed as milligrams (mg) by deciliter (dl) of plasma.

2.8. Determination of parasite weight and efficacy rate of treatments

After blood samples collection, animals were euthanized, and necropsy was carried out immediately thereafter. The cysts were removed from the peritoneal cavity. The weight of the cysts collected from each 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:

$$\text{Efficacy} = \frac{\text{XC} - \text{XT}}{\text{XC}} \times 100$$

where XC is the mean cysts weight in the untreated control group and XT is the mean cysts weight in the treated group.

2.9. Morphologic study

Samples of protoscoleces and cysts cultured in vitro as well as cysts recovered from mice involved in the efficacy study were processed for SEM as described by Elissondo et al. (2006, 2007).

2.10. Statistical analysis

The results obtained from in vitro studies are presented as protoscoleces and cysts viability (percent, arithmetic means ± SD). Log-rank test was used to assess the survival differences of protoscoleces and cysts after exposure to different concentrations of ABZ, 5-FU or ABZ + 5-FU. Statistical analyses were performed using the BioEstat 5.0 software (Ayres et al., 2007).

For the clinical efficacy study, cysts weights and enzyme data (reported as arithmetic mean ± SD) were compared by means of Kruskal–Wallis (non parametric ANOVA) followed by Dunn's multiple comparison test. These statistical analyses were performed using the Instat 3.0 Software (Graph Pad Software, CA, USA).

For all statistical comparisons, a P value less than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. In vitro effect of 5-FU + ABZ combination on protoscoleces and cysts

The survival of *E. granulosus* s. s. protoscoleces after exposure to ABZ, 5-FU and ABZ + 5-FU combination is shown in Fig. 2. Although all treatments had a protoscolicidal effect, the combination of 5-FU + ABZ provoked a stronger effect than the drugs alone.

Control protoscoleces cultured in medium 199 + DMSO remained viable (91.3 ± 1.3%) after 48 days of incubation. Treatment with 5-FU + ABZ produced dose- and time-dependent effects (Fig. 2). The maximum protoscolicidal effect was found with the combination of 10 μg/ml 5-FU + 10 μg/ml ABZ, reducing viability of protoscoleces to 22% after 12 days. In the same period of time, 10 μg/ml ABZ and 10 μg/ml 5-FU alone decreased the viability to 73% and 85%, respectively. At 24 days post-incubation, mortality was 100%. Loss of protoscolex viability in 5 μg/ml 5-FU + 5 μg/ml ABZ treated cultures became evident after 12 days, where the percentage value was 41.6 ± 8.4%, and reached 0% after 24 days. Even at the lowest concentration (1 μg/ml), the combination had a greater effect than 5-FU or ABZ alone at 10 μg/ml. In this case, the viability diminished to near 53% after 12 days of incubation. Viability was 0% at day 30.

The results of viability tests coincide with the tissue damage observed at the structural and ultrastructural level. Control cultures exhibited no morphological alterations during the whole incubation period (Fig. 3). The primary site of drug damage was the parasite tegument (Fig. 4). The first effects of the treatment with 5-FU + ABZ could be detected after 1 day post-incubation. They consisted in contraction of the soma region, formation of blebs on the tegument, loss of hooks and rostellar disorganization (Fig. 4a). The same alterations appeared later with 5-FU (3–4 days post-incubation) or ABZ (2–5 days post-incubation) incubated alone (Fig. 4a and b).

These results were confirmed on the ultrastructural level by SEM (Fig. 5). The damage provoked by the combination was faster and appeared to be broader than that observed with the drugs acting alone. After 12 days, protoscoleces incubated at the highest concentrations showed a complete loss of morphology (Fig. 5g). At this time, all protoscoleces incubated with 1 μg/ml 5-FU + 1 μg/ml ABZ revealed the

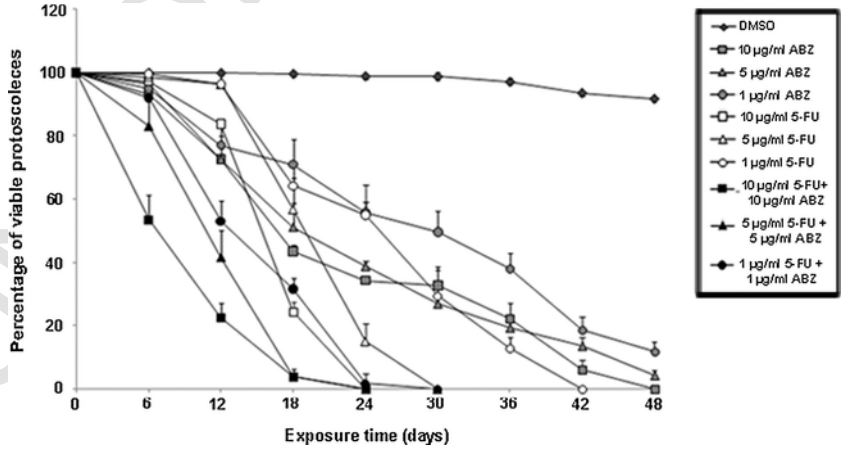

Fig. 2. Survival of *E. granulosus* s. s. protoscoleces after exposure to ABZ, 5-FU and to the combination of both drugs. Each point represents the mean percentage of vital protoscoleces from three different experiments (DMSO: dimethyl sulphoxide).

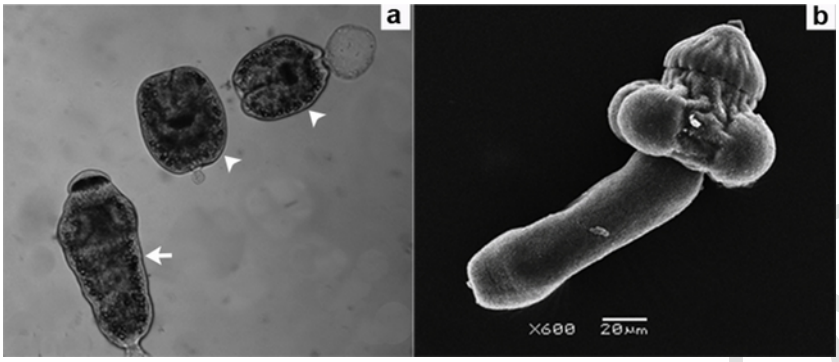

Fig. 3. Images of control *E. granulosus* s. s. protoscoleces after 12 days of incubation. a) Light microscopy (300×). Evaginated (arrow) and invaginated (arrowheads) protoscoleces; b) Scanning electron microscopy of an evaginated control protoscolex (600×).

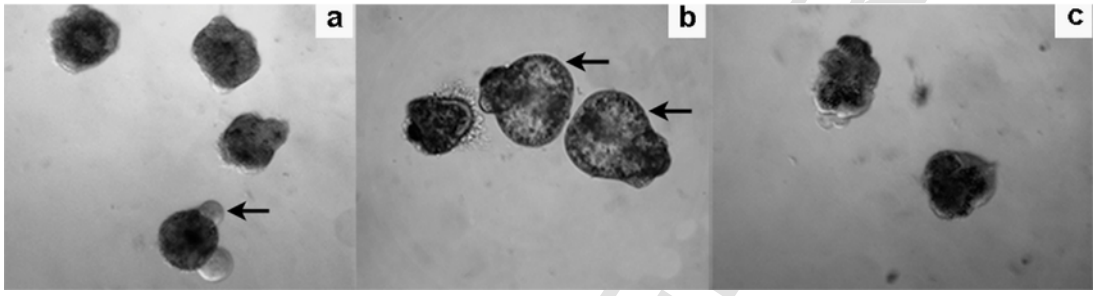

Fig. 4. Light microscopy of *E. granulosus* s. s. protoscoleces incubated in vitro with ABZ, 5-FU and 5-FU combined with ABZ. (a) Protoscoleces incubated with ABZ 10 μg/ml (400 ×). Note the presence of numerous blebs in the tegument (arrow); (b) Protoscoleces incubated with 5-FU 10 μg/ml. The tegument was markedly altered (arrows: vesiculated protoscoleces, 400×); (c) Altered protoscoleces incubated with 1 μg/ml 5-FU + 1 μg/ml ABZ (400×).

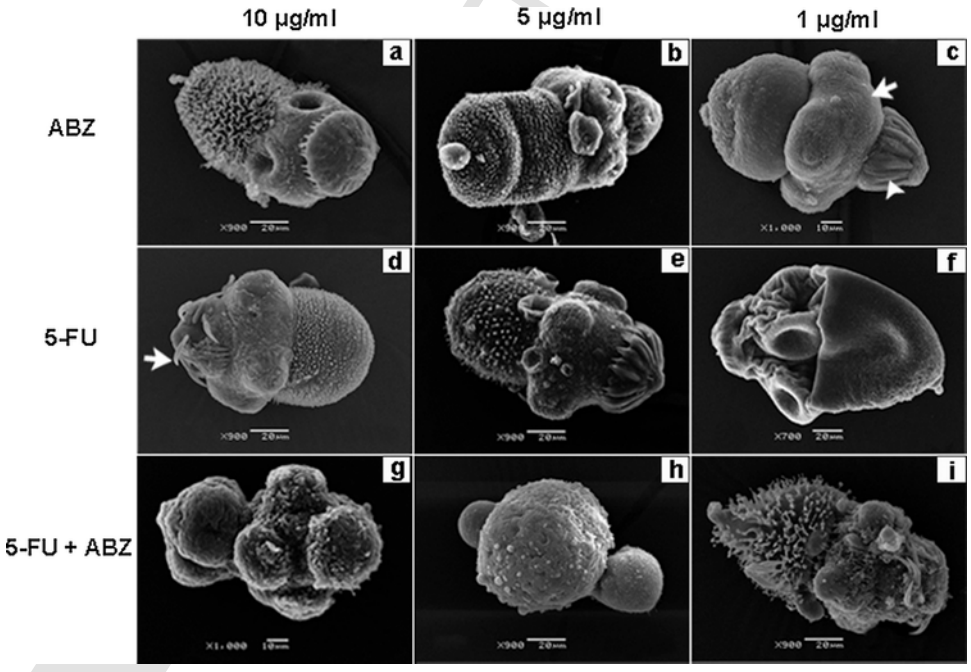

Fig. 5. Scanning electron microscopy images of *E. granulosus* s. s. protoscoleces incubated in vitro with ABZ, 5-FU alone and combined with ABZ during 12 days. (a–c) Protoscoleces exposed to ABZ. (a) Protoscolex markedly altered (10 μg/ml, 900×); (b) Protoscolex exposed to 5 μg/ml ABZ. The alteration of the tegument of the soma region, loss of hooks and sheeding of microtriches are evident (900×); (c) Observe the contraction of the soma region of a protoscolex incubated with 1 μg/ml ABZ. The rostellar disorganization (arrow) and shedding of microtriches could be observed (arrowhead) (1000×). (d-f) Protoscoleces incubated with 5-FU. (d) Contraction and alteration of the soma region are demonstrated. Observe the loss of hooks (arrow) (10 μg/ml, 900×); (e) Rostellar disorganization, shedding of microtriches and presence of blebs (5 μg/ml; 900×); (f) Vesiculated protoscolex after incubation with 1 μg/ml 5-FU (700×). (g-i) Protoscoleces incubated with 5-FU + ABZ combination. (g) Complete loss of the characteristic morphology (10 μg/ml 5-FU + 10 μg/ml ABZ; 1000×); (h) Protoscolex incubated with 5 μg/ml 5-FU + 5 μg/ml ABZ showing loss of hooks and absence of microtriches. Loss of morphology was evident (900×); (i) Protoscolex markedly altered. The digitiform tegumental extensions in the soma and the loss of hooks and microtriches in the scolex region are evident (1 μg/ml 5-FU + 1 μg/ml ABZ; 900×).

presence of tegumental alterations, rostellar disorganization, and complete shedding of microtriches (Fig. 5i).

The in vitro results of the incubation of hydatid cysts obtained from mice with the different drugs are shown in Table 1. The effect was more rapidly detected in cysts treated with the combination than when drugs were used separately. As shown in Fig. 6, co-treatment of murine cysts with 10 μg/ml 5-FU + 10 μg/ml ABZ resulted in the killing of 100% of cyst after 8 days compared with 40% with 10 μg/ml 5-FU and 80% with 10 μg/ml ABZ.

Inspection of control cysts by SEM revealed the typical features of *E. granulosus* s. s. metacestodes, with a distinct acellular outer laminated layer and an intact germinal layer comprised of a multitude of different, morphologically intact cell types (Fig. 7). The ultrastructural effect observed after co-incubation with 5-FU + ABZ was greater than that caused by 5-FU and ABZ alone.

3.2. Clinical efficacy study

The behavior and appearance of the animals were normal throughout the entire experiment period. Treatment with 5-FU or ABZ did not provoke a reduction in the body weight whereas in 5-FU + ABZ group the BWL was only 1.82%. Moreover, no statistical differences ($P > 0.05$) were found in AP, GGT and GTP activities and urea and Cr concentrations between control and treated mice (Fig. 8).

Hydatid cysts developed in all infected animals involved in the clinical efficacy study. Table 2 summarizes the cysts weights (mean ± SD) recorded after treatments on the different experimental groups involved in the efficacy study. There were no statistically differences ($P > 0.05$) between control groups. All treatments resulted in a statistically significant reduction on the cysts weight compared to those obtained for unmedicated mice. No difference was found in mean cysts weight between treated groups ($P > 0.05$) (Table 2). However, differences in the ultrastructural changes observed in the germinal layer of cysts recovered from treated groups were detected (Fig. 9).

The ultrastructural appearance of the germinal and laminated layers of cysts recovered from unmedicated mice was shown in Fig. 8. All cysts in the samples removed from control mice appeared turgid, showing no observable collapse of the germinal layer and no change in ultrastructure were detected (Fig. 8a and b). In contrast, the ultrastructural study of cysts developed in treated mice revealed changes in the germinal layer. However, the damage extension appears to be greater after co-treatment with 5-FU + ABZ compared to the monotherapy. Treatments with ABZ suspension or 5-FU caused reduction in cell number of the germinal layer altering their morphology (Figs. 8c–e). The combination showed a marked effect on the germinal layer. In all analyzed samples only cellular debris were observed (Fig. 8f).

4. Discussion

The development of new molecules for parasite targets is a process that requires a high investment in time and money (Hennessy, 1997). One approach to speeding up drug discovery is to find new uses for existing approved drugs. Drug repurposing is a useful strategy to accelerate the drug development process due to lower costs, reduced risk and decreased time to market due to availability of preclinical data (Panic et al., 2014). Repositioning also requires efforts to discover novel drug combinations that could produce synergistic and therefore superior effects compared with single compounds (Ferreira and Andricopulo, 2016).

As we mentioned, a promising starting point for the discovery of novel drugs to combat parasites is to examine available compounds developed against cancer for antiparasitic properties (Klinkert and Heussler, 2006). The in vitro activity of several antitumor drugs against *E. granulosus* s. l. was demonstrated ((Naguleswaran et al., 2006; Spicher et al., 2008a,b; Nicolao et al., 2014; Pensel et al., 2014; Yuan et al., 2016; Wang et al., 2017). However, the in vivo efficacy of many of these compounds does not correlate with the effect observed in vitro or has not been assayed to date.

The in vitro effect of the anti-cancer drug 5-FU on *E. granulosus* s. l. larval cells, protoscoleces and cysts has been previously reported (Pensel et al., 2014). In the present work, the in vivo effect of 5-FU alone or combined with ABZ in mice infected with the larval stage was evaluated. The development of treatments involving combinations of two or more drugs with different modes of action is an appealing approach to enhance its effectiveness, shorten treatment period and therefore decrease the toxicity (Lanusse et al., 2015).

In order to exclude inhibitory interactions, in vitro testing of drug combinations is mandatory (Reuter et al., 2010). As shown in the in vitro experiments, 5-FU + ABZ combination had a stronger effect against *E. granulosus* s. s. larval stage than that did both drugs alone. Even at the lowest concentration of 5-FU + ABZ combination (1 μg/ml), the reduction of the viability of protoscoleces and cysts was greater than that observed with drugs alone at 10 μg/ml. The results of viability test were confirmed at the ultrastructural level by SEM. The damage provoked by the combination was faster and appeared to be broader than that observed with the drugs acting alone. These data helped to justify the in vivo investigations assessing the therapeutic potential of the combination of 5-FU and ABZ in mice infected with *E. granulosus* s. s. metacestodes.

5-Fluorouracil is widely used in cancer treatment either alone or in combination with other drugs. For optimal effects in patients, 5-FU is given every week or daily as an i.v. bolus injection for five days at conventional doses of 10–20 mg/kg. Alternatively it is administered as a slow infusion, with infusion cycles of 24, 96 and even 120 h (Grem, 2000). The spectrum of toxicity associated with 5-FU treatment varies with dose, schedule, route of administration (Chabner and Longo, 2001). Intravenous bolus injection is commonly associated with gastrointestinal, haematological, neuronal, dermatological and cardiac adverse effects (Grem, 2000).

In the present work, 5-FU was given to mice weekly at the dose rate of 10 mg/kg once per week during 30 days. Based on the human equivalent dose formula (Nair and Jacob, 2016), this dose represents

Table 1
Time of appearance (days post-incubation) of different indicators of tissue damage on *E. granulosus* s. l. murine cysts, after its incubation with ABZ, 5-FU and 5-FU + ABZ, under in vitro conditions.

Parameters of the study	Days post-incubation									
	Drug concentrations (μg/ml)									
	Control	ABZ			5-FU			5-FU + ABZ		
		10	5	1	10	5	1	10	5	1
Loss of cyst turgidity	–	2	2	2	3	3	3	1	2	2
Appearance of collapsed cysts	–	3	3	3	4	4	4	2	3	3

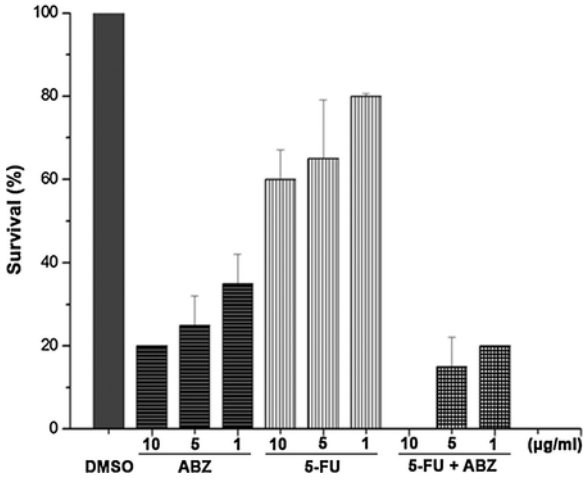

Fig. 6. Survival of *E. granulosus* s. s. murine cysts after 8 days of exposure to ABZ, 5-FU alone and combined with ABZ. Viability was measured on the basis of vesicle integrity (DMSO: dimethyl sulphoxide).

0.81 mg/kg of 5-FU. In addition, the used dose was six times less than the maximum tolerable dose in mice, using a weekly schedule for 4 weeks (Peters et al., 1986). According to the guide of care and use of laboratory animals, the behavior and appearance of the animals were normal throughout the treatments (National Research Council US, 2011). No statistical differences were found in AP, GGT and GTP activities and urea and Cr concentrations between control and treated mice. Moreover, 5-FU alone did not reduce the body weight of mice and, when it was co-administrated with ABZ the BWL was only 1.82%. This value was 10-fold less than the maximum established limit (Hares et al., 2013).

In agreement with other antitumor agents evaluated against *E. granulosus* s. l. (Nicolao et al., 2014; Wang et al., 2017), the in vitro effect of 5-FU was corroborated in the murine model of CE. 5-Fluorouracil has a short plasmatic half-life (10–20 min) as a consequence of a very rapid metabolism (Huang et al., 2016). Despite this limitation, 5-FU reduced significantly the weight of cysts recovered from treated mice in relation to control groups. In addition, the in vivo effect of 5-FU given weekly was comparable to that observed with ABZ suspension under a daily schedule during 30 days.

The mechanism of action of 5-FU has been well studied in several types of cancer cells. 5-FU is an analogue of uracil with a fluorine atom at the C-5 position instead of hydrogen and, therefore it is metabolized via the same metabolic pathways as uracil. As a pyrimidine analogue, it is transformed inside the cell into various cytotoxic metabolites: fluoro-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The active metabolites of 5-FU disrupt RNA synthesis (FUTP), can be directly misincorporated into DNA (FdUTP) and inhibit the action TS – a nucleotide synthetic enzyme (FdUMP) (Longley et al., 2003). Interestingly, TS is expressed in different stages of *E. granulosus* s. s. (GenBank accession number: EUB59800) (Zheng et al., 2013). Further biochemical and molecular studies are required in order to understand the mechanism of action of 5-FU in *E. granulosus* s. s. metacestodes.

Different drug combinations have been evaluated in mice infected with *E. granulosus* s.l. Combined treatments such as ABZ/ivermentin or ABZ/praziquantel has shown a synergism effect whereas co-administration of nitazoxanide/flubendazole did not improve the in vivo efficacy of the monotherapy (Ceballos et al., 2015; Moreno et al., 2001, 2002).

In the current study, co-administration of 5-FU with ABZ suspension did not enhance the in vivo efficacy of drugs alone calculated in relation to cysts weights. However, the ultrastructural alterations observed in cysts recovered from mice treated with the combination were greater than that provoked with the monotherapy. Pharmacokinetic or pharmacodynamics drug interactions are likely to occur, which should be further studied. By the other hand, the synergistic or antagonistic effect of the anticancer drugs combinations can depend on the ratio of

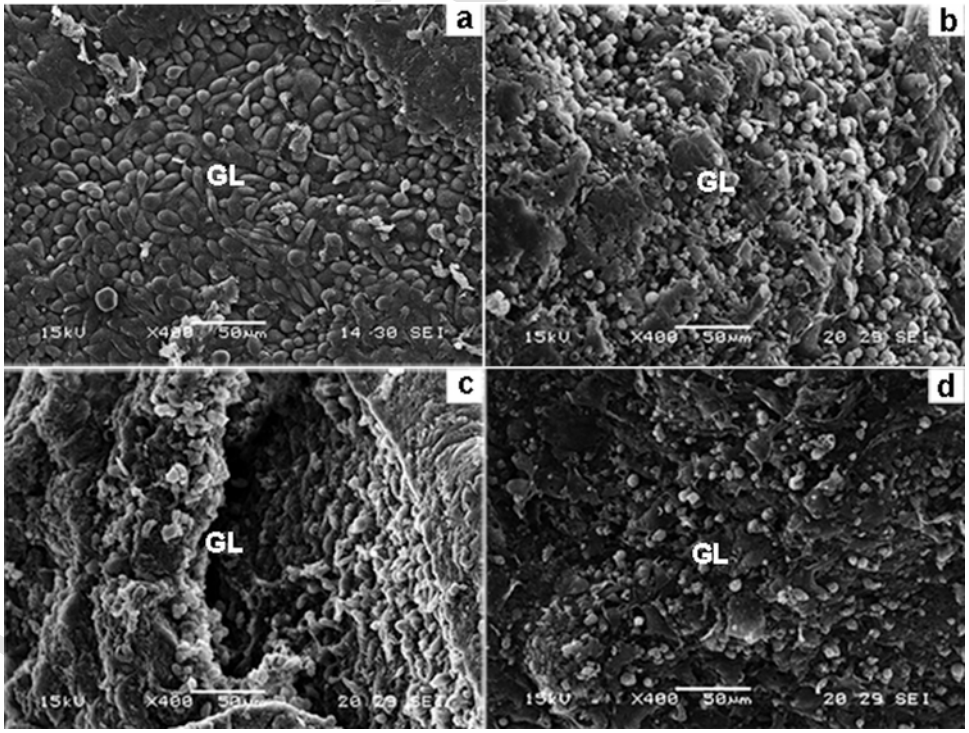

Fig. 7. Representative images of SEM of murine cysts incubated in vitro with ABZ, 5-FU and combined with ABZ during 6 days. (a) Control cyst with an intact germinal layer (gl, germinal layer; 400×); (b) Cyst incubated with 10 µg/ml ABZ. Germinal layer showing disintegrated areas (400×); (c) Cyst incubated with 10 µg/ml of 5-FU. The germinal layer is altered (400×); (d) Cyst incubated with 1 µg/ml 5-FU + 1 µg/ml ABZ. Note the extensive damage of the germinal layer. Only cellular debris could be observed (400×).

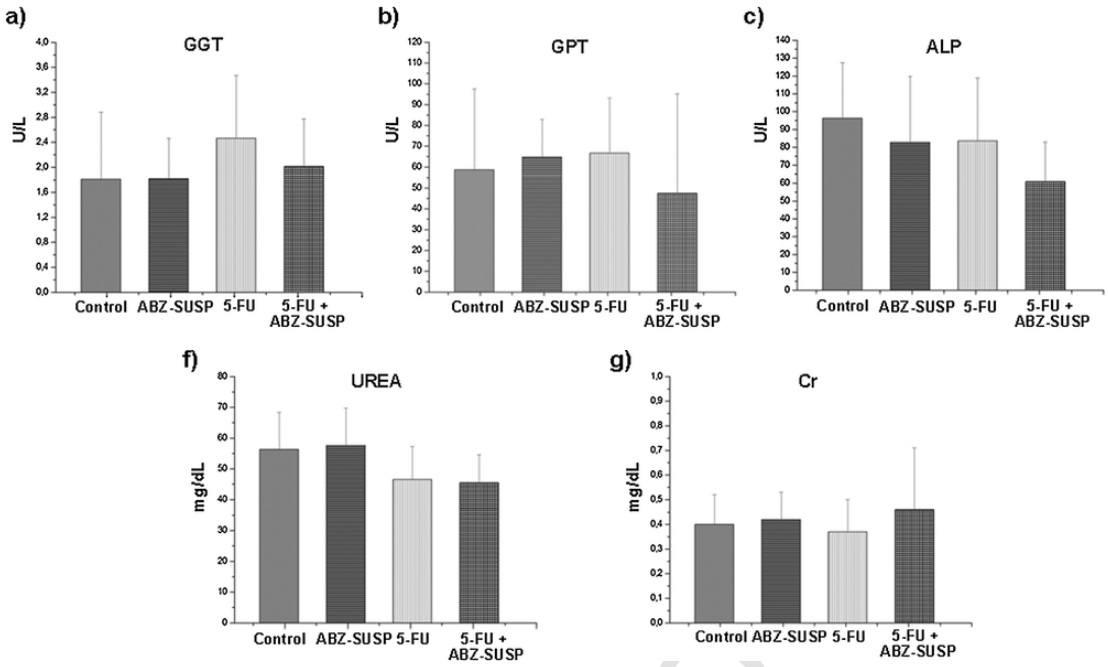

Fig. 8. Determination of enzymes gamma-glutamyl transpeptidase (GGT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) activities and urea and creatinine (Cr) concentrations in plasma of mice treated with ABZ suspension, 5-FU or the combination. There were no statistical differences between control and treatments ($P > 0.05$).

Table 2
Clinical efficacy study. Mean (±SD) weights (g) of the hydatid cysts recovered from infected mice treated with ABZ suspension (ABZ-SUSP), 5-fluorouracil (5-FU) or 5-FU + ABZ-SUSP. Treatments were performed after 5 months post-infection. ABZ suspension (5 mg/kg) was given via oral gavage once daily during 30 days. 5-FU (10 mg/kg) was injected once a week in the tail vein for 5 consecutive weeks.

	Clinical efficacy study	
	Wet weight (g) of cysts Mean ± SD	% of efficacy
Deionized water group	4.12 ± 2.63	
Saline group	4.50 ± 2.54	
ABZ-SUSP group	0.73 ± 0.71*	82.28
5-FU group	0.48 ± 0.30*	89.34
5-FU + ABZ-SUSP group	0.63 ± 0.38*	85.38

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

the combined agents (Harasym et al., 2010). Hence, it might be worthwhile the assessment of the in vivo effects of 5-FU and ABZ using other ratios.

In conclusion, we demonstrated the efficacy of 5-FU against murine experimental CE. Short periods of treatment were sufficient to achieve a pharmacological effect. Moreover, the ultrastructural alterations of cysts recovered from mice were greater when 5-FU was co-administrated with ABZ. Since 5-FU alone or in combination with ABZ did not cause toxic effect in mice, further in vivo studies will be performed by adjusting the dosage and the frequency of treatment.

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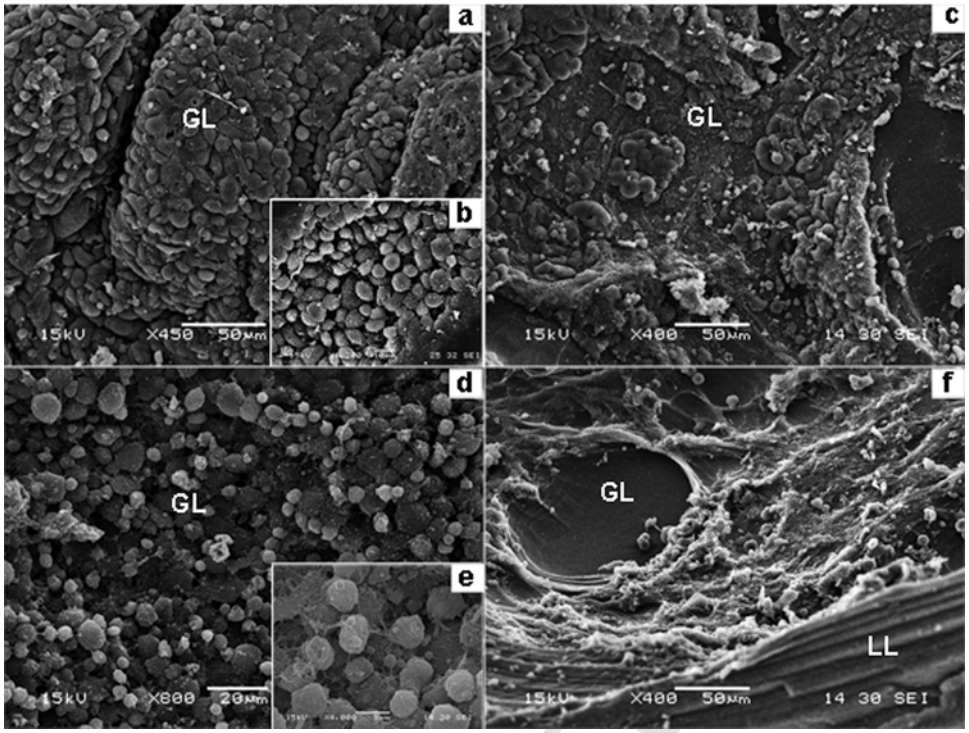

Fig. 9. Representative images of SEM images of cysts recovered from mice infected with *E. granulosus* s. s. treated with ABZ suspension, 5-FU or combination of 5-FU with ABZ suspension during the clinical efficacy study. Treatments began at 5 months post-infection. ABZ (5 mg/kg) was administered daily by oral gavage. 5-FU (10 mg/kg) was administered by i.v. injection once a week. The duration of the treatment was 30 days. (a) Cyst recovered from the control group (450×); (b) Detail of the different cell types. Note the intact morphology of germinal cells (1200×); (c) Cyst recovered from mice treated with the ABZ suspension. The germinal layer is altered with a clear reduction in cell number (400×); (d) Cysts recovered from mice treated with 5-FU. Note the loss of cells of germinal layer (800×); (e) Detail of the altered germinal cells (4000×); (f) Cyst recovered from 5-FU + ABZ-SUSP group. Germinal layer is markedly altered. Only debris of cells could be observed (400×).

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