

## **CHEMOPROPHYLACTIC ACTIVITY OF FLUBENDAZOLE IN CYSTIC ECHINOCOCCOSIS**

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## Abstract

Background: Cystic echinococcosis (CE) is an important public health problem worldwide. Flubendazole, administered as a suspension, has shown poor *in vivo* efficacy against CE in humans. However, flubendazole prepared as a solution caused a marked reduction in hydatid cysts developed in mice. The goal of the current work was to compare the chemoprophylactic effect of flubendazole formulated either as a hydroxipropyl- $\beta$ -cyclodextrin solution or as a carboxymethylcellulose suspension in secondary CE in mice. Methods: Balb/c mice were infected with *E. granulosus* protoscoleces. One day after infection the animals were allocated into three (3) different experimental groups: unmedicated control and treated at the time point of infection with flubendazole either prepared as a solution or suspension given twice a day during 15 days. Six months (6) post-infection, the animals were sacrificed to collect and weight parasite cysts. Cyst samples recovered from infected mice of each experimental group were prepared for both scanning and transmission electron microscopy. Results: Both flubendazole formulations induced a significantly reduction in cysts weight compared to those recovered from the unmedicated control animals. Both formulations showed similar flubendazole-induced ultrastructural morphological changes. Conclusion: Flubendazole offers a great potential to become a drug of choice in the preventive treatment of cystic echinococcosis.

**Keywords:** Flubendazole, cystic echinococcosis, cyclodextrin, chemoprophylaxis.

## Introduction

Human cystic echinococcosis (CE) is a zoonotic infection with a cosmopolitan distribution caused by the larval stage of the tapeworm *Echinococcus granulosus* [1]. The outcome of infection is the development of various size cysts [2] in any organ of the human body, most frequently in the liver (60-70%) and in the lung (20-30%) [3]. The clinical manifestations of CE are variable and determined by the site of location, size and condition of the cysts [3; 4]. The conventional surgery is still the chosen treatment for symptomatic cases of CE when the cysts feature and patients' status allow it. Surgical risks include those associated with any surgical intervention as well as those unique to echinococcosis such as anaphylaxis or secondary infection caused by the spillage of protoscoleces contained in the cysts fluid, with the potential to development into new cysts [5].

Routinely, surgery is accompanied by chemotherapy to reduce the risk of recurrence of CE. Furthermore, in patients where the surgery is inadequate, chemotherapy based on the use of benzimidazole anthelmintics is the treatment of choice [6]. However, 20-40% of cases do not respond favorably, without regression or complete disappearance of cysts [6]. The benzimidazole compounds that have shown acceptable efficacy are albendazole (ABZ) and mebendazole. ABZ is the most used drug [5], in spite of its variable intestinal absorption. The chemoprophylactic activity of benzimidazole therapy in humans used to prevent secondary infection during the surgery is unclear. However, reduction on cyst and protoscoleces viability has been reported [7]. Moreover, ABZ is usually recommended for 4 days before the procedure, continuing for at least 1 month after puncturing a lesion that was diagnosed as an *E. granulosus* cyst [8].

Flubendazole (FLBZ), an alternative benzimidazole methylcarbamate anthelmintic compound, has been evaluated for CE treatment in both mice [9] and man [10, 11]. In all cases, FLBZ administered orally as a suspension or tablets failed to affect *E. granulosus* cysts. However, FLBZ has shown to induce marked *in*

*vitro* damage on both protoscoleces and cysts of *E. granulosus* [9, 12]. Moreover, a clear reduction on cyst weight was observed after the administration of FLBZ prepared as a cyclodextrin -based solution in mice with cysts developed during 3 or 9 months, compared to those collected from the untreated mice [13]. The enhanced FLBZ systemic availability observed after its administration as a cyclodextrin-solution was correlated with an increased efficacy against secondary hydatid cyst developed in mice [13]. The FLBZ chemical structures and its most relevant pharmacological features are summarized in Figure 1. Nevertheless, further work is necessary to assess the potential preventive (chemoprophylactic) effect of FLBZ against secondary *E. granulosus* infection. The aim of the current work was to investigate the chemoprophylactic effect of FLBZ on mice infected with *E. granulosus* metacestodes, after its administration as a hydroxypropyl- $\beta$ -cyclodextrin (HPBCD) solution or a conventional carboxymethylcellulose (CMC) suspension. The weight of the recovered cysts was recorded. The drug-induced morphological changes were evaluated by both scanning and transmission electron microscopy in cysts obtained from untreated and treated (both formulations) mice.

## Materials and methods

### *Chemicals*

Janssen Animal Health (Beerse, Belgium) kindly provided pure analytical standards of FLBZ, and its metabolites, reduced-FLBZ (R-FLBZ) and hydrolyzed-FLBZ (H-FLBZ). The HPBCD was kindly supplied by Cargill Inc. (Hammond, IN, USA). CMC was from Anedra (Buenos Aires, Argentina).

### *FLBZ formulations*

A FLBZ solution was prepared by dissolution of 50 mg of pure FLBZ and 10 g of HPBCD in 100 mL of deionized water (pH 1.2). The pH was adjusted using hydrochloric acid (25 mM). The formulation was shaken during 48 h (40 °C) and then was filtrated through 0.45 µm filter (Whatman, NJ, USA). The final FLBZ concentration (0.5 mg/mL) was confirmed (n= 4) by HPLC. A FLBZ suspension (0.5 mg/mL) was prepared by addition of pure FLBZ in deionized water with CMC (0.5 % p/v, pH= 6.0) under shaking during 6 h. FLBZ suspension was vigorously shaken before its intragastric administration to mice. FLBZ formulations were freshly prepared every three days and maintained under refrigeration (3-5 °C).

### *Protoscoleces collection and culture*

Protoscoleces of *E. granulosus* were collected aseptically from liver hydatid cysts of infected cattle slaughtered in two abattoirs located in the southeast of Buenos Aires province, Argentina. Vitality was assessed by muscular movements (evaluated under light microscope), motility of flame cells and by the methylene blue exclusion test [14]. The culture protocols were carried out as described previously [15] using medium 199 (Gibco, Invitrogen, Buenos Aires, Argentina) supplemented with 100 IU penicillin, 100 µg/mL streptomycin, 4 mg/mL glucose and 20% (v/v) foetal calf serum.

### *Experimental animals*

Balb/C mice (6 months old at the starting of the experiments) were used to carry out the chemoprophylactic efficacy study. The animals were housed in temperature controlled ( $22 \pm 1$  °C), light- cycled (12h light/dark cycle) room. Food and water were given *ad libitum*. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina. <http://www.vet.unicen.edu.ar>

### *Experimental design*

#### *Chemoprophylactic efficacy study*

Balb/C mice (n= 42) were infected by intraperitoneal (i.p.) inoculation with 1500 *E. granulosus* protoescoleces/animal. At the time point of infection, the animals were allocated into three experimental groups (n=14) and treated as follow: Unmedicated control group (animals were treated with distilled water); FLBZ-solution treated group (animals were treated with the FLBZ-solution previously described), and FLBZ-suspension treated group (animals were treated with the FLBZ-suspension previously described). Treatments starting at the time point of infection were performed twice a day during 15 days by intragastric inoculation at the dose rate of 5 mg/kg. Six months after infection the animals were sacrificed by cervical dislocation and their peritoneal cavity was opened to remove the developed hydatid cysts. The weight of the cysts collected from each individual animal was recorded.

#### *Morphologic study*

For ultrastructure studies cyst samples recovered from each mouse were prepared for scanning (SEM) and transmission (TEM) electron microscopy to evaluate FLBZ-induced morphological alterations. The samples

were fixed with 3% glutaraldehyde in sodium cacodylate buffer for 24 h at 4°C. Then, several washes in cacodylate buffer were made. For SEM analysis, the specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50%-100%), and were finally immersed in hexamethyl-disilazane for 5 min, 1 h and then, overnight. They were then sputter-coated with gold (100 Å thickness), and inspected on a JEOL JSM-6460 LV scanning electron microscope operating at 15 kV. For TEM analysis, samples were post-fixed in 2% OsO<sub>4</sub> in cacodylate buffer, followed by several washes in water. They were dehydrated in a graded acetone series and subsequently embedded in Spurr's resin. Polymerization of the resin was carried out at 70°C overnight. Sections 700 angstrom thick were cut on a LKB ultramicrotome with diamond knife, stained with uranyl acetate saturated solution (45 min) and lead citrate (20 min) and examined with a JEOL 100 CXII transmission electron microscope at 80 kV.

## Results

All the infected mice (14/14) from the untreated control group developed hyaline hydatid cysts in the abdominal cavity, whereas in 4 (FLBZ suspension) and 5 (FLBZ solution) out of 14 treated mice the infection did not progressed. Moreover, the weights (g) of hydatid cysts (six months of development) recovered from artificially infected mice either in untreated (control) group or treated at the time point of infection with FLBZ formulated as s solution or suspension are shown in Table 1. Significant differences ( $P < 0.05$ ) were observed in the weight of the cyst recovered from unmedicated mice ( $2.42 \pm 0.91$  g) compared to both FLBZ-suspension ( $0.30 \pm 0.29$  g) and FLBZ-solution ( $0.64 \pm 0.62$  g) treated mice. No statistically significant differences were observed between cysts weight from both treated groups. The differences among experimental groups in cysts weight, showing the intra-group variability in individual mice are shown in Figure 2. The horizontal lines in the middle of the box represent the median for each group. Figure 3 shows representative images of structural appearance of the germinal and laminated layer after TEM and SEM evaluation of cysts removed from unmedicated mice, where all cysts appeared turgid, showing no observable collapse of the both layers, without ultrastructure alterations. Conversely, the TEM and SEM analysis of the cysts recovered from mice treated either with FLBZ-suspension (Figure 4) or FLBZ-solution (Figure 5), showed marked changes in the germinal layer with internal tissue extensively distorted, vacuolated areas, presence of abundant lipid droplets and residual bodies. This ultrastructural damage is consistent with a FLBZ-induced hydatid cyst degeneration.

## Discussion

Surgery is still the first treatment line for symptomatic cases of CE. However, the surgical procedure is not always possible since depends on the health status of the patient as well as on the type and location of the developed hydatid cysts. An important indication for benzimidazole treatment in human CE is the prevention of secondary infection during surgery, produced by the spillage of protoescoleces contained in the cysts. However, the role of the preoperative chemotherapy to prevent the risk of recurrence has not been extensively explored. ABZ, mebendazole and praziquantel were tested preoperatively with the objective of killing the protoscoleces before surgical treatment with unclear and variable results [7, 16]. More recently, Elissondo et al. [9] have reported the FLBZ activity against microcysts and protoscoleces of *E. granulosus* under *in vitro* conditions. The current experimental work was set out to investigate the chemoprophylactic effect of FLBZ on CE under *in vivo* conditions in a mice model.

HPBCD increased FLBZ water solubility, which accounted for its enhanced absorption and bioavailability in mice. This FLBZ-solution resulted in a significantly higher peak plasma concentration (C<sub>max</sub>) and area under the concentration vs time curve (AUC), compared to that obtained after the conventional FLBZ-suspension treatment [13]. Since the higher the concentration achieved at the tissue/fluid of parasite location, the greater the amount of drug reaching the target parasite [17], the increased FLBZ plasma concentrations facilitated by its formulation as a HPBCD was correlated with a significantly reduction on cyst weight. This efficacy patterns was observed in mice with cysts developed during 3 or 9 months compared to those collected from the untreated or FLBZ-suspension treated mice [13]. In the current experiment, similar FLBZ formulations and dose rates to those used by Ceballos et al., [13] were tested against protoscoleces inoculated in the peritoneal cavity of mice (drug treatment starting at the time point of infection), which simulates a cyst rupture during surgical practice.

After the oral administration of FLBZ to mice, both formulations (solution and suspension) demonstrated a preventive chemoprophylactic effect. Firstly, between 28 % (suspension) and 35 % (solution) of the FLBZ-treated mice did not develop any cyst. Oppositely, the presence of cysts was observed in all the mice from the unmedicated control group (Table 1). A deleterious drug effect on *E.granulosus* protoscoleces at the time of infection may help to explain the lack of cysts development observed in some animals of the FLBZ treated groups. Additionally, a significant ( $P<0.05$ ) reduction in cysts weight was observed in mice of both treated groups compared those recovered from untreated mice, indicating that FLBZ affected cyst development. The cyst weight recorded in FLBZ-solution or –suspension treated groups showed a reduction of 77 and 84 %, respectively. These results can not be considered as a completely effective chemotherapeutic effect. However, it is important to highlight that the course of the treatment involved only 15 days, which is a shorter period compared to other treatment schedules previously assayed [18, 19].

Previous studies [13] demonstrated a high efficacy of FLBZ against CE developed in mice (3 and 9 month of infection) after its administration as a HPBCD-solution, whereas the suspension formulation did not reach differences in efficacy with the unmedicated control group. However in the current experiment, no significant differences in cyst weight between both FLBZ formulations were observed. Since higher FLBZ plasma concentrations are achieved after its administration as a cyclodextrins-based solution, these results indicate that only low FLBZ concentrations may be required to achieve good efficacy against protoscoleces in the early stage of infection (chemoprophylactic effect). Thus, even the reduced systemic availability of FLBZ achieved after its administration as a drug suspension seems to be sufficient to affect cyst development in the early stage of infection, resulting in a chemopreventive activity similar to that obtained for the most bioavailable FLBZ-solution formulation.

The SEM and TEM evaluations revealed that FLBZ induces a number of characteristic alterations on the cyst germinal layer. However, differences in the ultrastructural changes observed in the germinal layer of cysts recovered from both FLBZ treated groups, were not observed. The ultrastructural changes induced by FLBZ were similar to that described for other benzimidazole compounds such as ABZ [20] and mebendazole [21], including distorted internal tissue, vacuolated areas, presence of abundant lipid droplets and residual bodies and loss of microtriches. The benzimidazole anthelmintics inhibits cytoskeletal tubulin polymerization affecting cell division, secretory transport systems and absorption. Most of the observed effects are related to increased vesiculation (including autophagic vacuoles), impairment/loss of microtriches, disorganized muscular fibers and loss of the integrity of the germinal layer. While the loss of microtriches are directly associated with failure of nutrient absorption and may induce loss of cyst viability, the large number of autophagosomes could be considered a response to general stress induced by the deleterious drug effect. The presence of many lipid droplets in the inner region of the germinal membrane is a common effect also observed following praziquantel [22] treatment, and could indicate general metabolic disruption of the cyst [23]. Interestingly, from the results obtained in the current experiment we can observe that FLBZ morphological effect on the cysts germinal layer remains several months after the end of FLBZ medication. Overall the work reported here demonstrates that *in vivo* treatment with FLBZ impaired hydatid cysts development. Between 20 and 35 % of the infected mice did not develop any cyst and there was a marked damage on the cysts recovered from treated animals, which was in a clear agreement with a reduction of the cysts weight collected from FLBZ-treated animals. In conclusion, the potential of FLBZ for use as a drug of choice in chemoprophylactic treatment of CE is promising. Further work is required to assess its potential for therapeutic use in human.

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## **Legends to Figures**

**Figure 1:** chemical structures and main pharmacological features of flubendazole.

**Figure 2:** box plot representation showing the comparative distribution of the weight (g) of hydatid cysts recovered from untreated and flubendazole (FLBZ) treated (suspension and solution) infected mice.

Both FLBZ treatments were given at 5 mg/kg, every 12 h over 15 days post infection.

A significant weight cysts reduction ( $P < 0.05$ ) was achieved in FLBZ treated animals (both treatments).

**Figure 3:** Scanning (SEM) and transmission (TEM) electron microscopy of hydatid cysts recovered from untreated control mice. **a)** SEM (x 650) image of hydatid cysts (gl: germinal layer). **b)** TEM (x 5000) image of hydatid cysts (ll: laminar layer; gl: germinal layer; mt: microtriches; g: glycogen; n: nucleus).

**Figure 4:** Representative images of scanning (SEM) and transmission (TEM) electron microscopy of hydatid cysts recovered from mice treated with flubendazole (FLBZ) prepared as a suspension. **a)** SEM (x 600) image of hydatid cysts. Alterations of the germinal layer (gl) can be appreciated . **b)** TEM (x 5000) image of hydatid cysts. The gl shows clear signs of alteration. Vacuoles (v), lipid droplets (l) in the laminar layer (ll), (mt: microtriches) are clear indication of flubendazole induced damage.

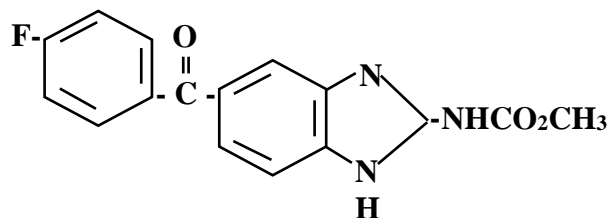
**Figure 5:** Representative images of scanning (SEM) and transmission (TEM) electron microscopy of hydatid cysts recovered from infected mice treated with flubendazole (FLBZ) prepared as a solution. **a)** SEM (x 600) image of hydatid cysts. The germinal layer (gl) is completely altered, only debris of cells

is observed. **b)** TEM (x 9000) image of hydatid cysts. The gl is completely disrupted. The presence of large lipid droplets (l) can be observed.

**Table heading**

**Table 1:** Weights (g) of hydatid cysts (six months of development) recovered from artificially infected mice either untreated (control) group or treated at the time point of infection with flubendazole (FLBZ) formulated as s solution or suspension (5 mg/kg, every 12 h over 15 days).

Figure 1



FLUBENDAZOLE (FLBZ)

1. Safe, broad-spectrum benzimidazole methyl carbamate anthelmintic
2. Widely used for nematode control in pigs, chicken, turkeys and game birds [24]
3. Parent FLBZ is extensively metabolized into reduced and hydrolyzed metabolites in different species [13, 25, 26, 27]
4. FLBZ formulation as cyclodextrin-based solutions increases its systemic bioavailability up to 5 folds in mice.
5. High *in vitro* activity against *E. granulosus* protoescoleces and microcysts [12] FLBZ-cyclodextrins based formulations increase its bioavailability in mice about 5 fold [13]
6. FLBZ induces a marked weight reduction of *E. granulosus* cysts developed in mice [13]

Figure 2

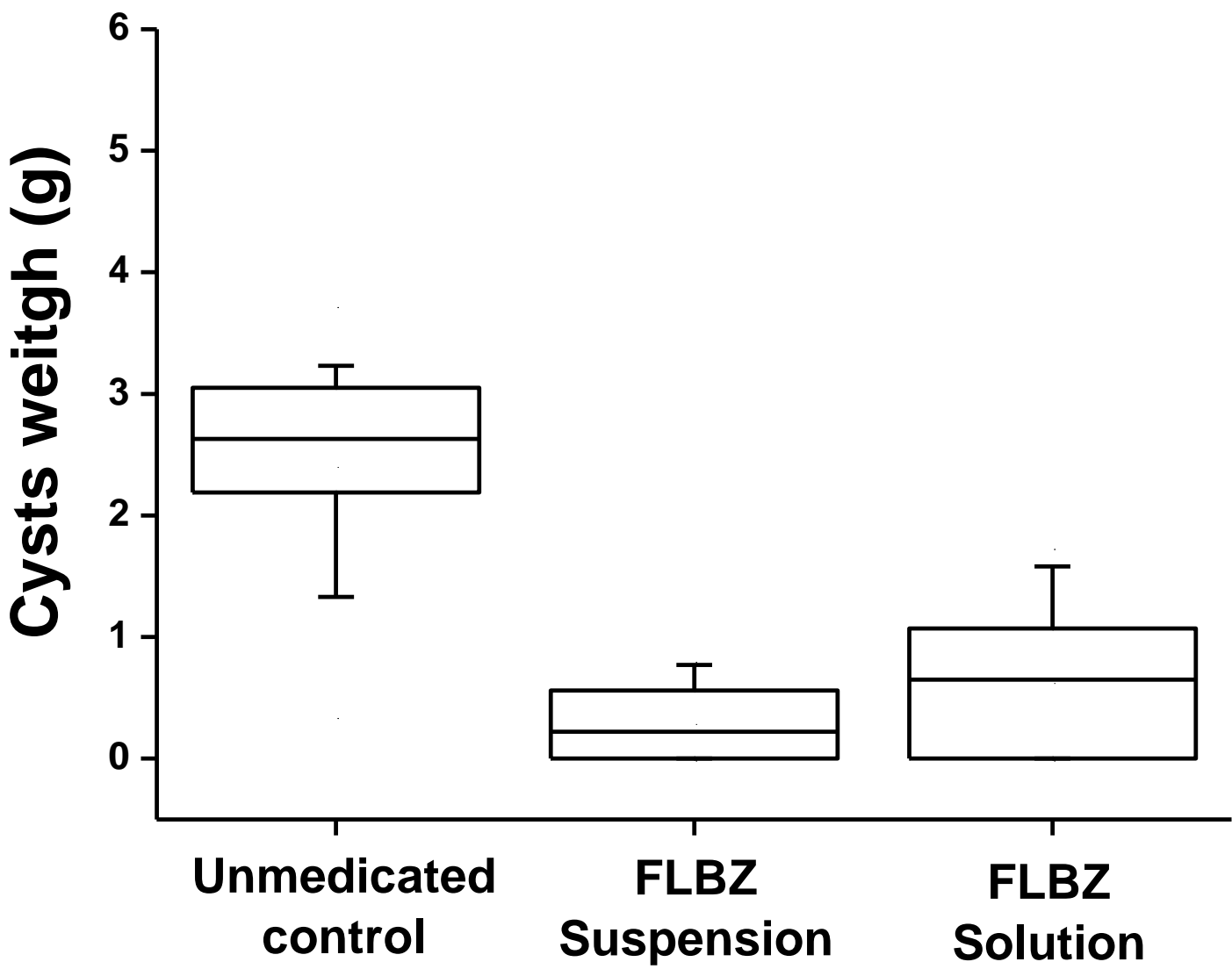


Table 1:

<b>Animal (#)</b>	<b>Untreated Control</b>	<b>FLBZ suspension</b>	<b>FLBZ solution</b>
1	1.33	0	0.65
2	0.35	0	0.46
3	2.63	0.77	0
4	2.41	0.22	1.07
5	2.19	0	1.02
6	1.38	0	1.11
7	3.05	0.56	0
8	3.23	0.22	1.58
9	3.03	0.42	1.06
10	2.83	0.37	0
11	3.22	0.58	0
12	3.73	0.81	1.74
13	2.24	0.13	0.23
14	2.19	0.12	0
<b>Mean <math>\pm</math> SD</b>	<b>2.42 <math>\pm</math> 0.91</b>	<b>0.30 <math>\pm</math> 0.29*</b>	<b>0.64 <math>\pm</math> 0.62*</b>

\* Statistically different from the untreated control group (P<0.05).

The zero (0) value indicates that no cysts were recovered from infected animals