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Flubendazole in cystic echinococcosis therapy: Pharmaco-parasitological evaluation in mice

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ABSTRACT

Cystic echinococcosis (CE) caused by the parasite *Echinococcus granulosus* is an important public health problem worldwide. Flubendazole has shown poor *in vivo* efficacy against CE in humans and mice. However, flubendazole causes marked *in vitro* damage on *E. granulosus* protoscoleces. The goals of the current work were: a) to compare the plasma pharmacokinetic behaviour of flubendazole formulated as a hydroxipropyl-β-cyclodextrin aqueous solution or as a carboxymethyl celullose suspension, both given by the oral route to mice, b) to compare flubendazole clinical efficacy in secondary CE in mice after its administration as both formulations, c) to evaluate the flubendazole-induced morphological changes in hydatid cysts recovered from infected mice treated with both drug formulations. Flubendazole administration as a solution resulted in significantly higher plasma maximum concentration (C_{max}) and area under the concentration–time curve (AUC) values compared to those obtained after the flubendazole-suspension treatment. This enhanced drug availability correlated with an increased efficacy against secondary CE in mice observed for the flubendazole-solution formulation, while the suspension formulation did not reach differences with the untreated control group. Similar ultrastructural changes were observed in cysts recovered from flubendazole (both formulations) treated mice after 3, 6 and 9 months of infection, although the damage extension was greater after treatment with the flubendazole-solution formulation.

1. Introduction

Echinococcus granulosus and *Echinococcus multilocularis* which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively, are the most important members of the genus *Echinococcus* due to their public health importance and geographical distribution [1,2]. CE or hydatidosis is in fact the most common presentation and probably accounts for more than 95% of the estimated global cases [3]. The disease affects humans as well as domestic livestock [4]. The dog is a definitive host, where adult tapeworms attached to the intestinal epithelium undergo sexual reproduction, leading to the development of eggs [4]. People became infected from ingestion of parasite eggs passed into the environment with faeces from definitive hosts. The outcome of the infection is the development of cysts, located in any organ/tissue [5], most frequently in the liver (60–70%) and lungs (20–30%) [6].

The induction of morbidity depends on the number, size, involved organ, localization of the cysts and the pressure of the cysts on surrounding tissues [7]. Furthermore, the possibility of cysts rupture and protoscoleces dissemination can result in either anaphylactic reaction or in a secondary CE caused by the spilled of large number of protoscoleces, which have the potential to differentiate into new hydatid cysts [7]. Currently, the disease treatment includes surgery and/or chemotherapy. Albendazole (ABZ), a benzimidazole (BZD) methylcarbamate compound, is the drug recommended in humans [8]. Although beneficial effects have been obtained in some studies, the response to treatment is frequently unpredictable [9]. The large variability observed in the therapeutic success of BZD anthelmintics on CE, may be explained by the host immunological status and/or the features of the cysts, including their size and location [7]. Furthermore, the poor/erratic gastrointestinal absorption is a common inconvenient for the systemic availability of orally administered BZD in most species [10]. Consequently, enhanced systemic availability of the parent drug/active metabolite obtained by increased drug absorption or by a reduced dosing interval, correlates with an improved antiparasitic effect, as it was demonstrated in several clinical studies [11–13].

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Flubendazole (FLBZ), an alternative BZD methylcarbamate compound, has been evaluated for CE treatment in both mice [14] and man [9,15]. FLBZ administered orally as a suspension or tablets failed to affect *E. granulosus* cysts. However, FLBZ has shown to produce marked *in vitro* damage on protoscoleces and cysts of *E. granulosus*. Interestingly, protoscoleces incubated with FLBZ were damaged faster than those cultured with ABZ or its active metabolite, ABZ-sulphoxide (ABZSO) [16]. It is likely that poor FLBZ gastrointestinal absorption has accounted for its low *in vivo* activity against CE. Therefore, the use of pharmacotechnical strategies to overcome this limitation may improve the *in vivo* efficacy of this drug.

It is well established that BZD dissolution is rate-limited by the acidic pH of the stomach [10]. As a consequence, the amount of drug absorbed above a minimum is constant and do not dependent on the administered dose. For example, the area under the concentration–time curves (AUC) was similar in dogs treated with a single administration of fenbendazole at different doses over a range of 25 to 100 mg/kg [17]. The use of complexing agents such as cyclodextrins (CD) is usually suggested [18] to increase the systemic bioavailability of poorly soluble compounds. Hydroxypropyl-β-cyclodextrin (HPBCD) increases ABZ water solubility [19]. Furthermore, an enhanced plasma availability of ABZ metabolites was reported after the administration of an ABZ-HPBCD formulation in sheep [20]. The goals of the current work were: a) to compare the plasma pharmacokinetic (PK) behaviour of FLBZ formulated as either an aqueous HPBCD-solution (FLBZ-solution) or an aqueous carboxymethyl cellulose (CMC)-suspension (FLBZ-suspension), both given by the oral route to mice; b) to compare FLBZ efficacy against secondary CE in mice after its administration as both formulations; c) to evaluate FLBZ-induced morphological changes in hydatid cysts recovered from infected mice treated with both drug formulations.

2. Materials and methods

2.1. Chemicals

Pure reference standards of FLBZ, reduced-FLBZ (R-FLBZ) and hydrolyzed-FLBZ (H-FLBZ) kindly provided by Janssen Animal Health (Beerse, Belgium) and oxibendazole (OBZ, Schering Plough Kenilworth, NJ, USA) were used for the validation of the analytical methodology. The solvents used for the chemical extraction and chromatographic analysis were HPLC grade (Baker, Inc., Phillipsburg, NJ, USA). Potassium phosphate (HPLC grade) and CMC were from Anedra (Buenos Aires, Argentina). Cargill Inc. (Hammond, IN, USA) kindly supplied us the HPBCD.

2.2. FLBZ formulations

The 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 by HPLC ($n=4$). 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. For the clinical efficacy studies, FLBZ formulations were freshly prepared every three days and maintained under refrigeration (3–5 °C).

2.3. Protoscoleces collection and culture

Protoscoleces of *E. granulosus* were aseptically collected 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 [21]. The culture protocols were carried out as described previously [22], 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.

2.4. Experimental animals

Male Balb/c mice (6 months old at the starting of the experiments) were used in both, the PK and the clinical efficacy trials. The animals were housed in temperature controlled (21 ± 2 °C), light-cycled (12 h light/dark cycle) room. Food and water were provided *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).

2.5. Experimental design

2.5.1. Pharmacokinetic study

Eighty eight (88) healthy mice were allocated into two groups of 44 animals each and treated with either, the FLBZ solution or the suspension. Both formulations were given orally at the same dose rate (5 mg/kg), using an intragastric tube. Blood samples obtained from sacrificed animals ($n=4$ per collection point) were collected in heparinized plastic tubes at the following times post-treatment: 5, 15 and 30 min, 1, 2, 3, 4, 6, 8, 10, 12 and 16 h. Blood samples were centrifuged at 2000 × *g* for 15 min and the recovered plasma was stored at −20 °C until analysis by HPLC.

2.5.2. Secondary infection with *E. granulosus* metacestodes. Efficacy study

Balb/c mice ($n=30$) were infected by intraperitoneal (i.p.) inoculation of 1500 protoscoleces/animal, suspended in 0.5 mL of medium 199. At three months post-infection, the animals were allocated into the following experimental groups (10 animals/group): (1) Unmedicated control group (animals received water as a placebo); (2) FLBZ-solution treated animals; (3) FLBZ-suspension treated mice. In all cases the treatments were performed by intragastric inoculation (0.3 mL/animal) every 12 h during 25 days. The FLBZ dose rate was 5 mg/kg. At the end of the treatment period, the animals were sacrificed by cervical dislocation and their peritoneal cavity was opened to remove the hydatid cysts. The weight of the cysts collected from each animal was recorded. The same experimental design was performed using infected mice with six and nine months of secondary infection with *E. granulosus* metacestodes.

2.5.3. Morphologic study

Cyst samples recovered from mice involved in the *efficacy study* (three, six and nine months of infection) were prepared for scanning (SEM) electron microscopy to evaluate the 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 performed. The specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%), and 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.

2.5.4. Analysis of FLBZ and its metabolites

Chromatography was performed on a Shimadzu HPLC equipment (Shimadzu Corporation, Kyoto, Japan), with two LC-10AS solvent pumps, an automatic sample injector (SIL-10A) with a 50 µL loop, an ultraviolet visible spectophotometric detector (UV) (SPD-10A)

reading at 292 nm, a column oven (Eppendorf TC-45, Eppendorf, Madison, WI, USA) set at 30 °C, and a CBM-10A integrator. Data and chromatograms were collected and analyzed using the Class LC10 software (SPD-10A, Shimadzu Corporation, Kyoto, Japan). The C18 reversed-phase column (5 µm, 250 mm × 4.6 mm) was Kromasil (Kromasil®, Sweden). Elution from the stationary phase was carried out at a flow rate of 1 mL/min using acetonitrile (40%) and potassium phosphate buffer (25 mM, pH 5.3, 40%), as a mobile phase.

Plasma samples (100 µL) were spiked with OBZ as internal standard (20 µL from stock solution of 5 µg/mL). After 5 min, plasma samples were mixed with 900 µL of water and the analytes were extracted using disposable C18 cartridges (Strata®, Phenomenex, CA, USA). The extraction procedures were as previously described [11]. Identification of FLBZ and its hydrolyzed (H-FLBZ) and reduced (R-FLBZ) metabolites was undertaken by comparison with the retention time of pure references standards. A complete validation of the analytical procedures for extraction and quantification of FLBZ, H-FLBZ and R-FLBZ in plasma was performed before starting the analysis of experimental samples. Retention times for H-FLBZ, R-FLBZ and FLBZ were 4.47, 5.79 and 9.69 min, respectively. The calibration curves for each analyte constructed by least squares linear regression analysis, showed good linearity with correlation coefficients ≥0.998. Mean absolute recoveries percentages for concentrations ranging between 0.01 and 1 µg/mL ($n = 5$) were 92.2 (H-FLBZ), 96.7 (R-FLBZ) and 91.1% (FLBZ) with CV of 5.49, 3.39, and 8.96%, respectively. The limit of quantification (0.01 µg/mL) was defined as the lowest measured concentration with a CV <20% an accuracy of ±20% and an absolute recovery ≥70%.

2.5.5. Kinetic analysis of the data

The concentration vs. time curves for FLBZ and/or its metabolites in plasma for each individual animal were fitted with the PK Solutions™ computer program (Summit Research Service, OH, USA). The peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration–time curve for each analyte. The area under the concentration–time curve (AUC) for FLBZ/metabolites was calculated by the trapezoidal rule [23], and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (h^{-1}).

2.5.6. Statistical analysis

PK parameters are presented as arithmetic mean ± SD. Student's *t*-test was used for the statistical comparison of the PK data obtained from the two experimental groups. A value of $P<0.05$ was considered statistically significant. For the clinical efficacy study, cyst weights (reported as arithmetic mean ± SD) were compared by analysis of variance (ANOVA) [24]. The Tuckey's range test was used to indicate the order of significance when a significant *F* value was obtained. A value of $P<0.05$ was considered statistically significant. The statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA).

3. Results

FLBZ and its hydrolyzed and reduced metabolites were recovered from mice plasma after the oral administration of FLBZ formulated as both a solution and a suspension. Fig. 1 shows the mean plasma concentration profiles of FLBZ, H-FLBZ and R-FLBZ after the oral administration of FLBZ-solution at 5 mg/kg to mice. FLBZ plasma concentration profiles were higher than those measured for its hydrolyzed or reduced metabolites. After its administration as a solution, FLBZ reached the peak plasma concentration at 42 ± 21.4 min post-treatment. R-FLBZ was the main metabolite detected in mice plasma after FLBZ treatment, reaching a C_{max} of 0.45 ± 0.49 µg/mL at 30 ± 14 min post-treatment (FLBZ-solution treatment). The H-FLBZ was the analyte measured at the lowest concentration

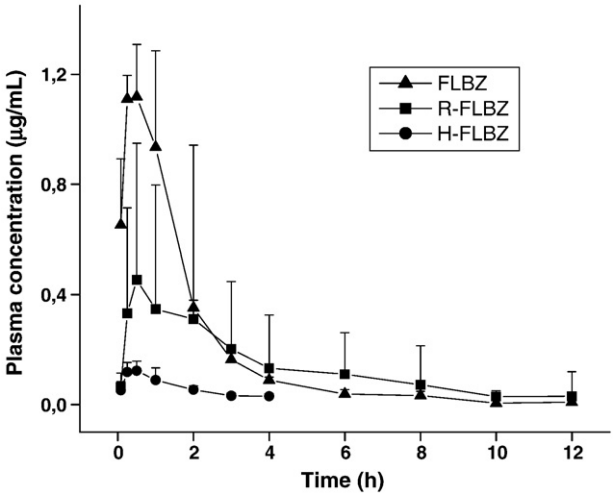

Fig. 1. Plasma concentration profiles (mean ± SD) of flubendazole (FLBZ) and its reduced (R-FLBZ) and hydrolyzed (H-FLBZ) metabolites at different times after the oral administration of FLBZ (5 mg/kg), prepared as a hydroxipropyl-β-cyclodextrin solution to healthy mice.

($C_{max} = 0.12 \pm 0.04$ µg/mL), being detectable only up to 4 h post-treatment. After the oral administration of FLBZ formulated as a suspension, the parent drug reach the C_{max} at 52 ± 15 min post-treatment. The peak plasma concentration (0.02 ± 0.01 µg/mL) for R-FLBZ was obtained at 30 ± 11.3 min post-treatment. Plasma concentrations of H-FLBZ under the limit of quantification were detected after the FLBZ-suspension administration. The comparative mean plasma concentration profiles of FLBZ after its oral administration as solution or suspension is shown in Fig. 2. Table 1 summarizes the plasma disposition kinetic data for FLBZ (mean ± SD) after its oral administration (both formulations) to mice. FLBZ administration as a solution resulted in a significantly ($P<0.05$) higher C_{max} (1.12 ± 0.001 µg/mL) compared to that obtained after the FLBZ-suspension treatment (0.04 ± 0.015 µg/mL) (Fig. 2). The enhanced FLBZ plasma profiles observed after FLBZ-solution treatment compared to that observed after FLBZ-suspension administration results in significantly ($P<0.05$) greater AUC values (2.17 ± 0.28 and 0.08 ± 0.05 µg.h/mL, respectively).

Hydatid cysts developed in all the infected animals involved in the efficacy studies. Table 2 summarizes the cyst weights (mean ± SD)

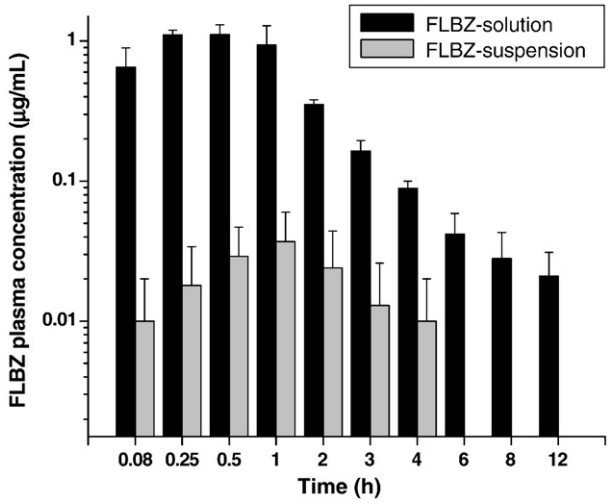

Fig. 2. Comparative flubendazole (FLBZ) plasma concentrations obtained after its oral administration as a hydroxipropyl-β-cyclodextrin-solution (FLBZ-solution), or a carboxymethyl celullose-suspension (FLBZ-suspension) to healthy mice.

Table 1
Pharmacokinetic parameters (mean ± SD) for flubendazole (FLBZ) after the oral administration (5 mg/kg) of FLBZ-solution and FLBZ-suspension to healthy mice.

Pharmacokinetic parameters	FLBZ formulations	
	FLBZ-solution	FLBZ-suspension
C_{max} (μg/mL)	1.12 ± 0.001	0.04 ± 0.02*
AUC_{total} (μg . h/mL)	2.17 ± 0.28	0.08 ± 0.05*
T_{max} (h)	0.70 ± 0.36	0.88 ± 0.25

C_{max}, peak plasma concentration; AUC_{total}, area under the concentration vs. time curve extrapolated to infinity; T_{max}, time to peak plasma concentration.
* Significantly different in comparison with the FLBZ-solution treated group ($P<0.05$).

recorded after treatments on the different experimental groups (Unmedicated control, FLBZ-solution and FLBZ-suspension treated groups) involved in the efficacy study at the different time post-infection (three, six and nine months). FLBZ treatment as a solution resulted in a reduction on the cysts weight compared to those obtained for unmedicated mice. This reduction resulted statistical significant ($P<0.05$) on cyst developed for three and nine months (see Table 2). Nevertheless, the treatment with the FLBZ suspension showed no significant differences in cyst weights at the three different times post-infection assessed in comparison with the cysts weight recorded for Unmedicated control group (Table 2).

Fig. 3 shows the ultrastructural appearance of the germinal and laminated layers after SEM analysis of cyst recovered from infected Unmedicated, FLBZ-solution and FLBZ-suspension treated mice (three months after infection). All cysts removed from Unmedicated control mice appeared turgid, showing no observable collapse of the germinal layer, in which no alteration in ultrastructure by SEM was observed. On the other hand, the ultrastructural study of cysts developed in mice treated with the FLBZ-solution or FLBZ-suspension revealed alterations in the germinal layer. The same ultrastructural changes were observed by SEM in cyst recovered from mice treated with the FLBZ-solution at 6 and 9 months post infection, as well as in cyst recovered from FLBZ-suspension treated mice (3, 6 and 9 months of infection). However, the damage extension appears to be broader after the FLBZ-solution compared to the suspension treatment.

4. Discussion

After the oral administration of FLBZ to mice (as both a solution and suspension), FLBZ parent drug was the main analyte found in plasma. R-FLBZ was the main metabolite recovered and low concentrations of H-FLBZ were measured. This drug/metabolites pattern was similar to that reported in sheep [25]. A fast FLBZ depletion from the bloodstream was observed after FLBZ administration (Fig. 1).

Drug particles must dissolve in the enteric fluids to facilitate absorption of the BZD molecule through the gastrointestinal (GI) mucosa. The dissolution rate of an enterally delivered compound influences the rate and extent of its absorption (systemic bioavailability), its maximal plasma concentration, its subsequent distribution to target tissues/parasites and its overall disposition kinetics. In the present experimental work, the use of a FLBZ HPBCD-based formulation induces drastic changes in FLBZ/metabolites plasma availability. HPBCD increased FLBZ water solubility, which accounted for its enhanced absorption and bioavailability in mice, resulting in a significantly higher (>450%) plasma C_{max} compared to that obtained after the FLBZ-suspension treatment ($P<0.05$). Since the higher the concentrations achieved at the tissue/fluid of parasite location, the greater the amount of

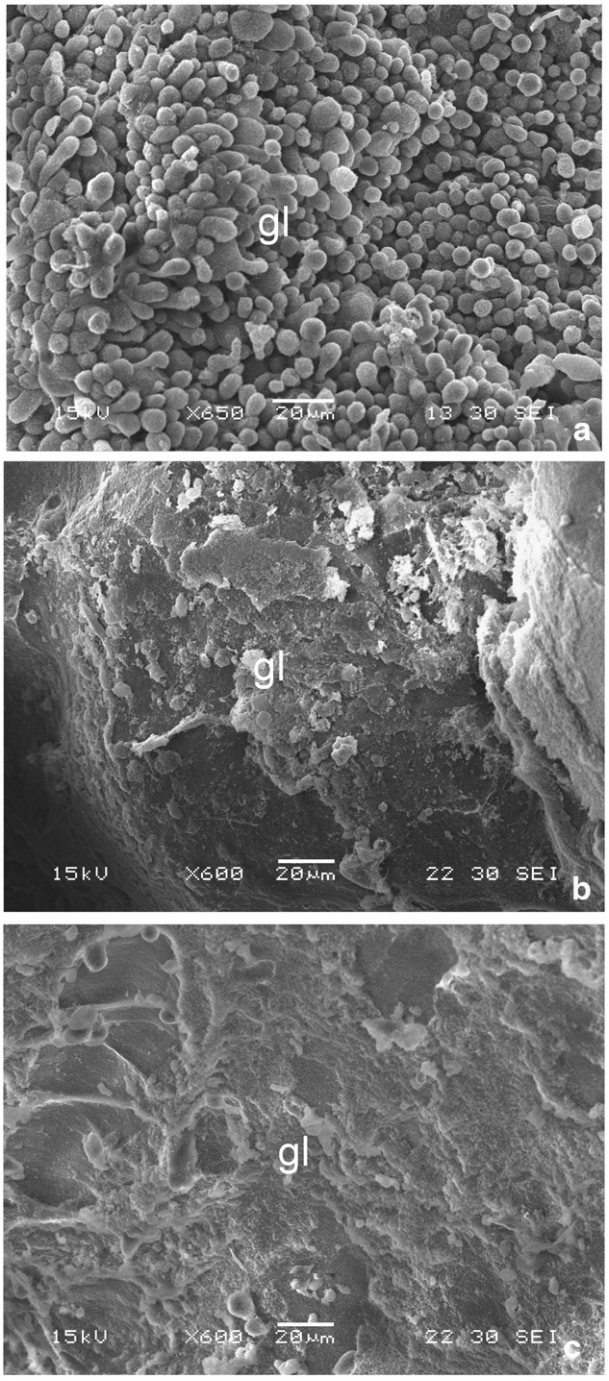

Fig. 3. Representative images of the scanning electron microscopy of hydatid cysts recovered from either Unmedicated control mice, FLBZ-solution or FLBZ-suspension treated mice. a) Scanning electron microscopy of cysts from unmedicated animals (gl: germinal layer; ×650). b) Scanning electron microscopy of cysts recovered from mice treated with the FLBZ-solution (5 mg/kg) after three months of infection. Alterations in the germinal layer (gl) and only debris of cells can be observed (×600). c) Scanning electron microscopy of cysts recovered from mice treated with the FLBZ-suspension formulation at 3 months post-infection. Alteration of the germinal layer (gl) can be appreciated, where only a few cells exhibit an intact morphology (×600).

Table 2
Mean (± SD) weights (g) of the hydatid cysts recovered at three, six and nine months post-infection from artificially infected mice from the Unmedicated control and from the flubendazole (FLBZ)-solution and FLBZ-suspension treated groups (5 mg/kg, every 12 h over 25 days).

Time post-infection (months)	Unmedicated control group	FLBZ-solution	FLBZ-suspension
3	0.27 ± 0.11[a]	0.06 ± 0.02[b]	0.40 ± 0.20[a]
6	1.16 ± 1.14[a]	0.18 ± 0.14[a]	1.20 ± 1.04[a]
9	11.0 ± 6.17[a]	1.07 ± 1.04[b]	8.50 ± 4.30[a]

Different letters indicate statistically significant differences ($P<0.05$) between experimental groups at the same time post-infection.

drug reaching the target parasite [26], the increased FLBZ bioavailability induced by HPBCD would impact on the amount of drug reaching the hydatid cysts and its overall antiparasitic effect.

Hydatid cysts developed in all the infected animals involved in the clinical efficacy studies. However, a large variation in cysts weights was observed, which agrees with previously reported results involving *E. granulosus* [11,27,28] and *E. multilocularis* [13,29,30] infection in laboratory animals. A drastic increase on cyst weight was observed along the three post-infection times in the Unmedicated control mice. Cyst weight increased about 300% between three and six months and 800% between six and nine months post-infection (Table 2), where the large metacestode mass infiltrated the whole abdominal cavity. A clear reduction on cyst weight was observed after the administration of FLBZ-solution in mice with cysts developed during three, six or nine months, compared to those collected from the Unmedicated control mice (Table 2). After administration of the FLBZ-solution, the mean cyst weight was reduced by 78, 84 and 90% (three, six or nine months post infection, respectively) in comparison to those recorded for unmedicated mice. However, these differences reached statistical significance only in three and nine months infected mice. In the six months-infected mice, the large individual variations (particularly observed within the Unmedicated control group) did not permit to obtain statistically significant differences, although there was a clear tendency in favour of the FLBZ-solution treatment. The limited GI absorption and consequent low systemic availability obtained following the FLBZ-suspension administration did not induce a significant cyst weight reduction compared to the unmedicated mice at any time post-infection (Table 2).

No ultrastructural changes (SEM analysis) were observed in the germinal membrane of cyst recovered from unmedicated mice with infection of three, six or nine months long. In contrast, the germinal layer of cysts recovered from FLBZ-solution and FLBZ-suspension treated mice was markedly altered (Fig. 3). Debris of cells could be observed (FLBZ-solution treatment) or only few cells with an intact morphology (FLBZ-suspension treatment). The ultrastructural changes observed in the germinal membrane of cysts recovered from FLBZ treated animals after both treatments (FLBZ-solution or suspension) were identical. However, the damage extension appears to be broader after the FLBZ-solution compared to the suspension, which may correlate with the observed advantageous drug concentration profiles in the bloodstream and consequently, with enhanced drug availability at the site of cyst location.

In conclusion, the improved kinetic behaviour of FLBZ administered as a solution resulted in a higher drug exposure of the hydatid cysts, which enhanced FLBZ clinical efficacy on CE developed in mice. FLBZ may be a suitable drug for treating CE and the use of pharmacotechnically-based strategies would be helpful to improve clinical efficacy of BZD anthelmintics to treat the hydatid disease.

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