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PII: S0304-4017(17)30534-4
DOI: https://doi.org/10.1016/j.vetpar.2017.12.022
Reference: VETPAR 8544

To appear in: *Veterinary Parasitology*

Received date: 9-1-2017
Revised date: 27-12-2017
Accepted date: 29-12-2017

Please cite this article as: Pensel, Patricia, Paredes, Alejandro, Albani, Clara M., Allemandi, Daniel, Bruni, Sergio Sanchez, Palma, Santiago D., Elissondo, María C., Albendazole nanocrystals in experimental alveolar echinococcosis: enhanced chemoprophylactic and clinical efficacy in infected mice.Veterinary Parasitology https://doi.org/10.1016/j.vetpar.2017.12.022

Albendazole nanocrystals in experimental alveolar echinococcosis: enhanced chemoprophylactic and clinical efficacy in infected mice

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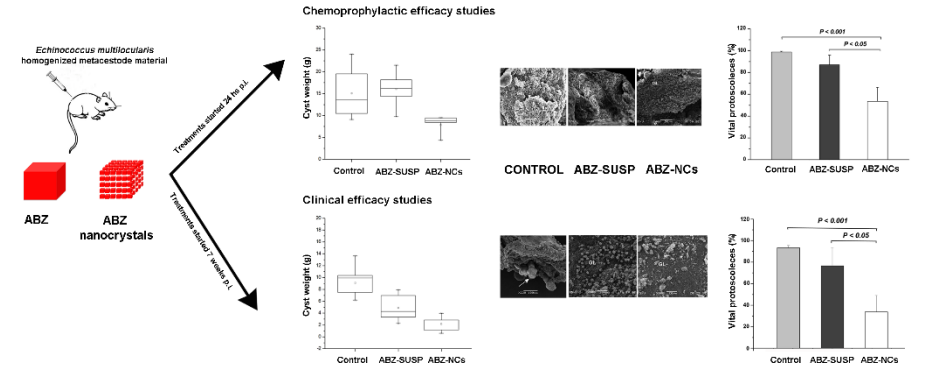
Graphical abstract



Highlights

- Albendazole nanocrystals (ABZ-NCs) had greater chemoprophylactic and clinical efficacy than albendazole.
- These outcomes could be a consequence of the increase in the systemic availability of albendazole sulfoxide.
- ABZ-NCs are a suitable alternative for treating echinococcosis in humans.

Abstract

Human alveolar echinococcosis is caused by the fox tapeworm *Echinococcus multilocularis* and is usually fatal if left untreated. Medical treatment with albendazole (ABZ) remains an effective option. However, due to its low aqueous solubility, ABZ is poorly and erratically absorbed following oral administration resulting in low drug levels in plasma and liver distribution. Thus, there arises the need to find a simple, efficient and scalable method to produce new ABZ formulations with increased bioavailability. Bearing this in mind, ABZ nanocrystals (ABZ-NCs) appears to be a

useful tool to achieve this goal. The aim of the current study was to investigate the chemoprophylactic and clinical efficacy of an ABZ-NC formulation on mice infected with *E. multilocularis*. In the chemoprophylactic efficacy study, mean weight of the cysts recovered from the ABZ-NC group was 50% lower than that recorded from untreated mice, whereas the treatment with ABZ suspension did not show preventive effect. The viability of protoscoleces isolated from ABZ-NC treated mice was significantly lower than control groups. In the clinical efficacy studies, both ABZ formulations resulted in a reduction in the mean weight of the cysts obtained from mice, however only the treatment with the nanosuspension revealed significant differences ($P < 0.05$) compared to the control groups. Treatment with ABZ-NCs reduced the weight of the cysts by 77% and the viability of their protoscoleces to 34%. All these results coincided with the tissue damage determined at the ultrastructural level. The enhanced chemoprophylactic and clinical efficacy of ABZ-NCs observed in this study could be attributed to an increase in the oral bioavailability of the drug. In a next step, we will characterize the cyst concentration profile after the administration of ABZ-NCs in mice infected with *E. multilocularis*.

Keywords: Alveolar echinococcosis, *Echinococcus multilocularis*, albendazole, nanocrystals.

1. Introduction

Alveolar echinococcosis (AE) is a serious helminthic zoonosis caused by the metacestode stage of *Echinococcus multilocularis*. This parasitic infection can lead to severe damage to the human liver, lungs and other organs, and is potentially lethal disease when left untreated. Infection of intermediate host such as rodents or accidentally humans is initiated by oral uptake of infectious eggs, which contain the oncosphere larva. After hatching in the host intestine, oncospheres penetrate the intestinal wall and use the blood and lymphatic system for dissemination. They typically invade the liver, where they develop into the metacestode stage. *E. multilocularis* metacestodes proliferate asexually by forming small daughter vesicles, leading to a parasite mass that exhibits tumor-like properties and progressively infiltrates the neighboring tissue (Kern, 2010).

The major treatment option for AE is radical surgery accompanied by pre- and post-operative medical treatment with albendazole (ABZ). Operative resection of lesions is frequently incomplete because of the tumor-like proliferation and the multilocular aspect of the parasite. In cases where surgery is not possible, medical treatment with benzimidazole methyl carbamates such as mebendazole and ABZ remains an effective option. ABZ is the most common and effective antiparasitic drug for AE treatment (Brunetti et al., 2010). However, due to its low aqueous solubility (0.2 µg/ml in water at 25 °C), ABZ is poorly and erratically absorbed following oral administration resulting in low drug levels in plasma and liver distribution (Jung et al., 1998; Daniel-Mwambete et al., 2004). Consequently, this compound has to be administrated at high or multiple doses in order to provide therapeutic concentrations, causing adverse effects in some cases (Brunetti et al., 2010). On the other hand, the poor water solubility of ABZ offers

only few formulation possibilities, limiting the administration routes (Alanazi et al., 2007).

Whereas permeability is an intrinsic drug property that is hard to modify, different techniques have been developed which can improve ABZ water solubility and dissolution rate, such as the formulation of solid dispersions (Castro et al., 2012), oil/water emulsion (Mingjie et al., 2002, Shuhua et al., 2002), incorporation into liposomes (Wen et al., 1996), cyclodextrin complexes (Palomares-Alonso et al., 2010), co-grinding (Vogt et al., 2008) and chitosan-microspheres (Abulaihaiti et al., 2015). Several clinical studies have demonstrated that enhanced systemic availability of the parent drug/active metabolite obtained by increased drug absorption correlates with an improved antiparasitic effect (Wen et al., 1996; Mingjie et al., 2002; Shuhua et al., 2002; Dvoroznáková et al., 2004; Ceballos et al., 2008; Palomares-Alonso et al., 2010; Pensel et al., 2014; 2015; Abulaihaiti et al., 2015).

Most of the strategies described above have shown limited success in the improvement of ABZ bioavailability (Paredes et al., 2016). Besides, these formulation approaches have relatively low drug loading efficiency and manufacturing process are complicated (Su and Yeo, 2012). In this context, the formulation of drug nanocrystals (NCs) has emerged as a very promising tool for the formulation of poorly soluble drugs.

By definition, drug NCs are nanoparticles being composed of 100% drug, being generally stabilized by surfactants or polymeric steric agents and according to the definition of nanoparticles the mean particle size is below 1 μm. NCs are generally produced in an aqueous medium as nanosuspensions and then, further solvent removal might be necessary in order to obtain redispersible powders (Müller et al., 2011).

According to the Noyes–Whitney and Ostwald–Freundlich equations, a decrease in particle size lead to an increase in the specific surface, enhancing the drug dissolution

rate and the saturation solubility (Gao et al., 2012). Moreover, the increase in the contact area leads to an increase in the mucoadhesiveness of nanomaterial. Therefore drug NCs display series of benefits in oral application of poorly soluble drugs, including improved oral absorption, higher bioavailability, rapid action onset, reduced fed/fasted state variability and reduced intersubject variability (Shegokar and Müller, 2010).

Recently, Paredes et al. (2016) produced ABZ-NCs with a final particle size of approximately 500 nm by an optimized methodology. ABZ formulated as powdered self-dispersible NCs presented a high redispersion capacity, as well as enhanced saturation concentration and dissolution rate. For orally administered drugs, optimization of these two properties is relevant (Paredes et al., 2016). Oral administration of ABZ-NCs increased the drug plasma levels in dogs and healthy mice (Paredes et al., 2017; 2018). Moreover, this improved pharmacokinetic performance observed for ABZ-NC formulation correlated with an improved in vivo therapeutic response against a model intestinal haematophagous nematode parasite in dogs (Paredes et al., 2018). The aim of the current study was to investigate the chemoprophylactic and clinical efficacy of an ABZ-NC formulation on mice infected with *E. multilocularis* metacestodes.

2. Materials and methods

2.1. Chemicals

ABZ was purchased from Todo Droga, Argentina. The stabilizer Poloxamer 188 (P188) was obtained from Rumapel, Argentina. All assays were performed using Milli-Q® water (Merck Millipore, USA).

2.2. Nanocrystal formulation

The ABZ nanosuspension was prepared by high pressure homogenization and further water removal was carried out by spray drying as described by (Paredes et al., 2016). Briefly, 5 g of ABZ and 5 g of P188 were ground in a mortar; water (190 ml) was gradually added until a homogeneous suspension was obtained. Afterwards, the sample was processed in a high pressure homogenizer by 30 cycles at 1200 bar (Avestin C5 Emulsiflex®, Canada). Samples were cooled using a heat exchanger with counter-flow cold water (5 °C) during the homogenization process. The obtained nanosuspension was spray-dried (Mini Spray Dryer B-290, Büchi Labortechnik AG, Switzerland) using a two-fluid nozzle with a cap orifice diameter of 1.5 mm with the operating conditions being: atomization air (L/h): 819, aspiration (m^3/h): 30, temperature (°C): 45 and feed pump (ml/min): 2. The obtained powder of ABZ: P188 (1:1) was used for the efficacy studies.

2.3. ABZ formulations

ABZ suspension (0.5 mg/ml) was prepared by dispersion of ABZ pure standard in MilliQ water (pH=7.0) under shaking (12 h). The nanosupension (0.75 mg/ml) was prepared by dissolution of ABZ-NCs in Milli-Q® water (pH=7.0) under shaking. The formulations were vigorously shaken before its intragastric administration to mice.

2.4. Ethic statement

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 (body weight 25 ± 5 g) 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.

2.5. Parasite material

All experiments were carried out using parasite isolate 8065 (kindly provided by Klaus Brehm, Institute for Hygiene and Microbiology, University of Würzburg). Cystic mass dissected from experimentally infected female CF-1 mice was pressed through a metal tea strainer and the suspension obtained was washed several times with an antibiotic solution and maintained in the same solution overnight before intraperitoneal inoculation into mice (Albani et al., 2015).

2.6. Experimental design

Eighty mice were infected by intraperitoneal inoculation with 0.5 ml of homogenized metacestode material. Two different experimental designs were conducted: a chemoprophylactic efficacy study and a clinical efficacy study.

2.6.1. Chemoprophylactic efficacy study

Twenty four hours after the infection, mice (n = 40) were allocated into 4 experimental groups (10 animals/group) and treated as follows: a) Control group, animals receiving Milli-Q® water as a placebo; b) Blank-NC group, animals receiving excipients in Milli-Q® water; c) ABZ suspension treated group; d) ABZ-NC treated group. Treatments were performed daily for 30 days by intragastric administration at the ABZ dose of 5 mg/kg. Eleven weeks after infection, mice were euthanized, and necropsy was carried out immediately thereafter.

2.6.2. Clinical efficacy studies

At 7 weeks post-infection, mice (n =40) were allocated into the following experimental groups (10 animals/group) and treated as follows: a) Control group, animals receiving Milli-Q® water as a placebo; b) Blank-NC group, animals receiving

excipients in Milli-Q® water ; c) ABZ suspension treated group; d) ABZ-NC treated group. Dose rate and administration regimen were the same as those used for chemoprophylactic efficacy study. At the end of the treatment period, animals were euthanized, and necropsy was carried out immediately thereafter.

2.7. Determination of efficacy rate of treatments

At necropsy in both studies, the peritoneal cavity was opened, and the hydatid cysts were carefully removed. The weight of the cysts collected from each animal was recorded using an analytical balance (Denver Instrument, USA). The treatment efficacy was evaluated by the mean cyst weight, viabilityof protoscoleces and the ultrastructural study of cysts and protoscoleces.

2.7.1. Efficacy of treatments based on the weight of cysts from infected mice

The efficacy of treatments, was calculated by use of the following formula:

$$\% \text{ of efficacy} = \frac{(X_C - X_T)}{X_C} * 100$$

where X_C is the mean cyst weight in the untreated control group and X_T is the mean cyst weight in the treated group.

2.7.2. Isolation of protoscoleces and viability test

The isolation of protoscoleces of parasite tissue from each animal (medical and unmedicated animals) was performed as described by Albani et al. (2015). Protoscoleces viability was performed using the methylene blue exclusion test (Elissondo et al., 2007). Dead protoscoleces stain blue and those that are alive exclude the dye and remain clear.

2.7.3. Morphologic study

Samples of cysts collected from each mouse were processed for scanning and transmission electron microscopy (SEM and TEM) as described by Elissondo et al. (2007). Moreover, samples of protoscoleces recovered from cysts obtained from each individual animal were processed for SEM, using the protocol described in Elissondo et al. (2008).

2.8. Statistical analysis

Data are reported as arithmetic mean (±SD) and statistical analysis was performed using the Instat 3.0 Software (GraphPad Software, CA, USA). Cyst weight and protoscolex viability data were compared by means of Kruskal–Wallis test (non parametric ANOVA) followed by Dunn's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chemoprophylactic efficacy study

Metacestodes developed in all infected animals involved in the chemoprophylactic efficacy studies. There was no statistically significant difference ($P > 0.05$) between the mean cyst weight of control groups (i.e. control and Blank-NC groups). Treatment with ABZ suspension did not cause any alteration on the weight of cysts recovered from mice compared to the control groups (16.04 ± 3.89 g; $P > 0.05$). Conversely, after oral administration of ABZ nanosuspension, the weights of cysts were significantly lower than those obtained in unmedicated groups ($P < 0.01$; ABZ-NC group: 7.84 ± 2.15 g).

Cysts isolated from mice treated with ABZ-NCs lacked the typical structure when observed macroscopically. They lacked turgidity and showed a lax appearance of the cyst wall (data not shown).

All cysts in the samples removed from control mice appeared turgid, no changes in the ultrastructure were detected (Fig. 1a). TEM analysis of cysts recovered from control groups revealed typical features of *E. multilocularis* metacestodes, with a distinct acellular outer laminated layer and a germinal layer without alterations, clearly delineated microtriches, and abundant glycogen storage cells (Fig. 1b).

In contrast, SEM and TEM analysis of the parasite tissue recovered from mice treated either with ABZ suspension (Figs. 1 c and d) or ABZ-NCs (Figs. 1 e and f) revealed changes in the germinal layer. Studies by SEM revealed that the germinal layer of metacestodes from treated mice lost the multicellular structure features. TEM analysis of parasite tissue obtained from ABZ suspension treated mice exhibited the presence of vacuolated areas and distorted microtriches (Fig. 1 d). Metacestodes recovered from ABZ-NC treated group showed marked alteration in the germinal layer with internal tissue distorted, increased numbers of cytoplasmic vacuoles and depleted glycogen storage cells. Moreover, mitochondria in the tegument were increased in numbers and they were more electron-dense (Fig 2 f).

The viability of *E. multilocularis* protoscoleces isolated from treated mice is shown in Fig. 2A. Following the isolation of protoscoleces from the control groups, the methylen blue viability test revealed that > 99% of the recovered parasites were still viable (Control group: 99.06 ± 0.8%, Blanck-NC group: 99.55± 0.72%). There was no statistically significant difference between the viability of protoscoleces recovered from control and ABZ suspension treated groups ($P > 0.05$; ABZ suspension: 87.1 ± 8.9%). In contrast, viability of protoscoleces from ABZ-NC group was significantly lower than control groups ($P < 0.001$; ABZ-NCs: 53.84 ± 12.81%).

The results of viability test coincide with the tissue damage determined at the ultrastructural level. Protoscoleces isolated from control mice showed typical

morphology (Figs. 2B a and b). Rostellar disorganization and contraction of soma region could be observed in some protoscoleces from ABZ suspension treated group (Fig. 2B c). On the other hand, all protoscoleces recovered from ABZ-NC treated mice lacked normal structure showing contraction of soma region, rostellar disorganization and disruption of microtriches pattern (Fig. 2B d and e).

3.2. Clinical efficacy study

Table 2 summarizes the cyst weights (mean ± SD) recorded after treatments of the different experimental groups involved in the efficacy study. There were no statistically differences between the control groups ($P > 0.05$). Although treatment with ABZ suspension provoked a clear reduction of the cyst weight, no significant differences were found compared to control groups (4.9 ± 2.2 g, $P > 0.05$). In contrast, ABZ-NC treatment resulted in a statistically significant reduction (2.17 ± 1.24 g, $P < 0.01$) on the cyst weight compared to those obtained for unmedicated mice.

The ultrastructural study of cysts developed in mice treated with both ABZ formulations revealed changes in the germinal layer. However, the damage of extension appears to be greater after ABZ-NC treatment. Observations by SEM revealed only debris of cells in cysts recovered from the nanosuspension group while few cells with an intact morphology were detected for the ABZ suspension treatment group (Figs. 3 c and e). TEM analysis of cysts recovered after administration of ABZ suspension showed slight utrastructural alterations with the presence of vacuolated areas (Fig. 3 d). On the other hand, treatment with ABZ-NCs provoked a marked alteration in the germinal layer with the internal tissue extensively distorted and vacuolated areas (Fig. 3 f).

There were no statistical differences in the viability of protoscoleces from control groups ($P > 0.05$, Control group: 93.41 ± 2.77%; Blanck-NC group: 95.96 ±

3.30%). Both formulations of ABZ showed a protoscolicidal effect (Fig. 4A). Despite the reduction of protoscoleces viability to 74.65 ± 16.5% upon ABZ suspension treatment, no significant differences ($P < 0.05$) were found respect to the control groups. On the other hand, administration of ABZ-NCs provoked a clear protoscolicidal effect decreasing the viability to 34% ($P < 0.001$). Viability values correlated with SEM studies (Fig. 4B). Some protoscoleces of the ABZ suspension treated group showed alterations in the tegument, soma contraction and rostelar disorganization (Fig. 4B c). The same alterations were detected in all protoscoleces of the ABZ-NC group (Fig. 4B d).

4. Discussion

The slow dissolution rate of ABZ leads generally to a poor and erratic absorption from the gastrointestinal tract, affecting its pharmacologic efficacy in clinical use (Castro et al., 2012). A slight increase in drug solubility has been shown to have major influence on intestinal absorption and resultant pharmacokinetic behaviour (Ceballos et al., 2008). Therefore, the use of pharmacotechnical strategies to overcome this limitation may markedly improve the treatment of systemic infections such as echinococcosis. In the present study, we demonstrated that oral administration of ABZ formulated as NCs improved the in vivo efficacy of the drug in mice infected with *E. multilocularis*.

Recently, Paredes et al. (2016) developed self-dispersible ABZ-NCs with a final particle size of approximately 500 nm. ABZ-NCs were able to improve the pure ABZ physico-chemical properties such as saturation concentration and dissolution rate.This nano-sized formulation enhanced the plasma availability of ABZ sulfoxide in dogs and healthy mice (Paredes et al., 2017, 2018).

The enlarged specific surface of the drug nanoparticles, allows its rapid dissolution in the acid conditions of the stomach, therefore a high drug concentration gradient between the gastrointestinal tract and blood vessel is produced. This phenomen improves absorption to a large extent and leads to a high bioavailability (Gao et al., 2008). In addition, the adhesiveness to the gut wall is also conducive to a high bioavailability by prolonging residence and contact time in the gastrointestinal tract (Gao et al., 2008).

Available scientific evidence indicates that higher ABZ bioavailability correlates with an improved antiparasitic effect (Ceballos et al., 2008; Palomares-Alonso et al., 2010; Pensel et al., 2014; 2015). The improved pharmacokinetic performance observed for ABZ-NCs correlated with an enhanced in vivo therapeutic response against *Ancylostoma caninum* in dogs (Paredes et al 2018). In accordance with these studies, we found that ABZ-NCs improved significantly the althelmictic effect of the drug in mice infected with *E. multilocularis*. We suggest that this enhanced of ABZ-NC activity could be attributed to an increase of the drug bioavailability.

In the chemoprophylactic efficacy study, the mean weight of cysts recovered from ABZ-NC group was 50% lower than that recorded from untreated mice, whereas the treatment with ABZ suspension did not show preventive effect. The ultrastructural changes observed by electron microscopy included the loss of the characteristic multicellular appearance of the germinal layer, distorted internal tissue with presence of vacuolated areas and depletion of glycogen reserves. This results coincide with the observed alterations in other chemoprophylactic studies againts the murine model of CE (Urrea-Paris et al., 2001; Ceballos et al., 2010; Moazeni et al., 2014; Pensel et al., 2014; Maggiore et al., 2015). Moreover, the viability of *E. multilocularis* protoscoleces isolated from ABZ-NC treated mice was significantly lower than that from control

groups. A deleterious drug effect on protoscoleces at the time of infection could explain the development of anomalous cysts and protoscoleces (Maggiore et al., 2015).

In the clinical efficacy study, both ABZ formulations resulted in a reduction in the mean weight of the cysts obtained from treated mice, however only the treatment with the nanosuspension revealed significant differences compared to the control groups. Ultrastructural studies showed an evident effect of the nanosuspension against the germinal layer of metacestodes. On the other hand, we found a positive correlation between the mean cyst weight and the protoscolex viability recorded in each treatment group. Oral administration of ABZ-NCs (5 mg/kg) reduced the weight of the cysts by 77% and the viability of their protoscoleces to 34%. Treatment with the ABZ suspension, despite being administered at the same dose, reduced the weight of the cysts by 46% and did not have a significant effect on the viability of protoscoleces.

In contrast to other nanoparticle systems, drug NCs consist mainly of pure active drugs (Junyaprasert and Morakul, 2015). Drug NCs exhibit many advantages including high efficiency of drug loading, easy scale-up for manufacturing, relatively low cost for preparation and long-term stability. All these advantages allowed the introduction of several NC-based formulations into the market (Zhou et al., 2016). Moreover, NCs can be administered using different routes, such as oral, parenteral, ocular, dermal and pulmonary delivery (Gao et al., 2012).

The safety of ABZ has been extensively investigated in a wide range of antiparasitic indications. At low dose, the incidence of adverse experiences is low. At the higher doses and more prolonged exposure used in the treatment of echinococcosis, there is an increase in the number and severity of adverse experiences. Adverse effects include nausea, vomiting, diarrhea, dizziness, headache, neutropenia, liver toxicity, alopecia and others. These reactions, reversible upon cessation of treatment, have been noted in a

minority of patients treated with ABZ (Horton, 1997, Brunetti et al., 2010). The ABZ-NC formulations would allow administering lower doses of drug in the treatment of echinococcosis, with the consequent reduction in side effects. When ABZ-NCs were administered to naturally parasitized dogs, it was possible to achieve the same efficacy using a dose which was four times lower than a commercial formulation (Paredes et al., 2018)

In conclusion, we consider that ABZ-NCs are an excellent nanotechnology strategy for the chemotherapy of infectious diseases such as echinococcosis. In a next step, we will characterize the cyst concentration profile after the administration of ABZ-NCs in mice infected with *E. multilocularis*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Melendez and Dr. Sebastián González (SENASA, Argentina). This work was supported by the PICT 15 No. 0717 (ANPCyT, Argentina), PIP 0029 (CONICET, Argentina), and Universidad Nacional de Mar del Plata (Grant EXA 672/14 and EXA 769/16), Argentina.

References

Abulaihaiti, M., Wu, X.W., Qiao, L., Lv, H.L., Zhang, H.W., Aduwayi, N., Wang, Y.J. Wang, X.C., Peng, X.Y., 2015. Efficacy of albendazole-chitosan microsphere-based treatment for alveolar echinococcosis in mice. PLoS Negl. Trop. Dis. 9, e0003950.

Alanazi, F.K., El-Badry, M., Ahmed, M.O., Alsarra, I.A., 2007. Improvement of albendazole dissolution by preparing microparticles using spray-drying technique. Sci. Pharm. 75, 63–79.

Brunetti, E., Kern, P., Vuitton, D.A., 2010. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 114, 1–16.

Castro, S.G., Sánchez Bruni, S., Urbizu, L.P., Confalonieri, A., Ceballos, L., Lanusse, C.E., Allemandi, D.A., Palma, S.D., 2012. Enhanced dissolution and systemic availability of albendazole formulated as solid dispersions. Pharm. Dev. Technol. 18, 434–442.

Ceballos, L., Elissondo, C., Moreno, L., Dopchiz, M., Sánchez Bruni, S., Denegri, G., Alvarez, L., Lanusse, C., 2008. Albendazole treatment in cystic echinococcosis: pharmacokinetics and clinical efficacy of two different aqueous formulations. Parasitol. Res. 103, 355–362.

Ceballos, L., Elissondo, C., Sánchez Bruni, S., Confalonieri, A., Denegri, G., Alvarez, L., Lanusse, C., 2010. Chemoprophylactic activity of flubendazole in cystic echinococcosis. Chemother. 565, 386–392.

Daniel-Mwambete, K., Torrado, S., Cuesta-Bandera, C., Ponce-Gordo, F., Torrado, J.J., 2004. The effect of solubilisation on the oral bioavailability of three benzimidazole carbamate drugs. Int. J. Pharm. 272, 29–36.

Dvoroznáková, E., Hrcková, G., Borosková, Z., Velebný, S., Dubinský, P., 2004. Effect of treatment with free and liposomized albendazole on selected immunological parameters and cyst growth in mice infected with *Echinococcus multilocularis*. Parasitol. Int. 53, 315–325.

Elissondo, M.C., Ceballos, L., Dopchiz, M., Andresiuk, V., Alvarez, L., Sánchez Bruni, S., Lanusse, C., Denegri, G., 2007. In vitro and in vivo effects of flubendazole on *Echinococcus granulosus* metacestodes. Parasitol. Res. 100, 1003–1009.

Elissondo, M.C., Albani, C.M., Gende, L., Eguaras, M., Denegri, G., 2008. Efficacy of thymol against *Echinococcus granulosus* protoscoleces. Parasitol. Int. 57, 185–90.

Gao, L., Zhang, D., Chen, M., 2008. Drug nanocrystals for the formulation of poorly soluble drugs and its application as a potential drug delivery system. J. Nanopart. Res. 10, 845 –862.

Gao, L., Liu, G., Ma, J., Wang, X., Zhou, L., Li, X., 2012. Drug nanocrystals: in vivo performances. J. Control. Release 160, 418–430.

Horton, R.J. 1997. Albendazole in treatment of human cystic echinococcosis: 12 years of experience. Acta Trop. 64, 79–93.

Jung, H., Medina, L., Garcia, L., Fuentes, I., Moreno-Esparza, R., 1998. Absorption studies of albendazole and some physicochemical properties of the drug and its metabolite albendazole sulphoxide. J. Pharm. Pharmacol. 50, 43–48.

Junyaprasert, V.B., Morakul, B., 2015. Nanocrystals for enhancement of oral bioavailability of poorly water-soluble drugs. Asian J. Pharm. 10, 13–23.

Kern, P., 2010. Clinical features and treatment of alveolar echinococcosis. Curr. Opin. Infect. Dis. 23, 505–512.

Maggiore, M., Pensel, P.E., Denegri, G., Elissondo, M.C., 2015. Chemoprophylactic and therapeutic efficacy of thymol in murine cystic echinococcosis. Parasitol. Int. 64, 435–440.

Mingjie, W., Shuhua, X., Junjie, C., Bin, L., Cheng, F., Weixia, S., Hotez, P., 2002. Albendazole-soybean oil emulsion for the treatment of human cystic echinococcosis: Evaluation of bioavailability and bioequivalence. Acta Trop. 83, 177–181.

Moazeni, M., Larki, S., Oryan, A., Saharkhiz, M.J., 2014. Preventive and therapeutic effects of *Zataria multiflora* methanolic extract on hydatid cyst: An in vivo study. Vet. Parasitol. 205, 107 –112.

Müller, R.H., Gohla, S., Keck, C.M., 2011. State of the art of nanocrystals – Special features, production, nanotoxicology aspects and intracellular delivery. Eur. J. Pharm. Biopharm. 78, 1–9.

National Research Council US. 2011. Guide for the Care and Use of Laboratory Animals, 8th edition. National Academies Press (US), Washington (DC), ISBN-13: 978–0-309–15400-0 ISBN-10: 0–309-15400–6.

Palomares-Alonso, F., González, C.R., Bernad-Bernad, M.J., Montiel, M.D.C., Hernández, G.P., González-Hernández, I., Castro-Torres, N., Pinzón Estrada, E., Jung-Cook, H., 2010. Two novel ternary albendazole-cyclodextrin-polymersystems: dissolution, bioavailability and efficacy against *Taenia crassiceps* cysts. Acta Trop. 113, 56–60.

Paredes, A.J., Llabot, J.M., Sánchez Bruni, S., Allemandi, D., Palma, S.D., 2016. Self-dispersible nanocrystals of albendazole produced by high pressure homogenization and spray-drying. Drug Dev. Ind. Pharm. 42, 1564–1570.

Paredes, A.J., Litterio, N., Dib, A., Allemandi, D.A., Lanusse, C., Sánchez Bruni, S., Palma, S.D. 2018. A nanocrystal□based formulation improves the

pharmacokinetic performance and therapeutic response of albendazole in dogs. J. Pharm. Pharmacol. 70: 51–58. Doi 10.1111/jphp.12834.

Paredes, A.J., Sánchez Bruni, S., Allemandi, D., Lanusse, C., Palma, S.D. 2017. Albendazole Nanocrystals with Improved Pharmacokinetic Performance in Mice. Ther. Deliv. In press.

Pensel, P.E., Castro, S., Allemandi, D., Sánchez Bruni, S., Palma, S.D., Elissondo, M.C., 2014. Enhanced chemoprophylactic and clinical efficacy of albendazole formulated as solid dispersions in experimental cystic echinococcosis. Vet. Parasitol. 203, 80–86.

Pensel, P.E., Ullio Gamboa, G., Fabbri, J., Ceballos, L., Sanchez Bruni, S., Alvarez, L.I., Benoit, J.P., Palma, S.D., Elissondo, M.C., 2015. Cystic echinococcosis therapy: Albendazole-loaded lipid nanocapsules enhance the oral bioavailability and efficacy in experimentally infected mice. Acta Trop. 152, 185–194.

Shegokar, R., Müller, R.H., 2010. Nanocrystals: industrially feasible multifunctional formulation technology for poorly soluble actives. Int. J. Pharm. 399, 129–139.

Shuhua, X., Jiqing, Y., Mingjie, W., Pieying, J., Fanghua, G., Junjie, C., Wei, J., Hotez, P., 2002. Augmented bioavailability and cysticidal activity of albendazole reformulated in soybean emulsion in mice infected with *Echinococcus granulosus* or *Echinococcus multilocularis*. Acta Trop. 82, 77–84.

Sun, B., Yeo, Y., 2012. Nanocrystals for the parenteral delivery of poorly water-soluble drugs. Curr. Opin. Solid. St. M. 16, 295–301.

Urrea-París, M., Casado, N., Moreno, M., Rodriguez-Caabeiro, F., 2001. Chemoprophylactic praziquantel treatment in experimental hydatidosis. Parasitol. Res. 87, 510–512.

Vogt, M., Kunath, K., Dressman, J.B., 2008. Dissolution improvement of four poorly water soluble drugs by cogrinding with commonly used excipients. Eur. J. Pharm. Biopharm. 68, 330–337.

Wen, H., New, R.R., Muhmut, M., Wang, J.H., Wang, Y.H., Zhang, J.H., Shao, Y.M., Craig, P.S., 1996. Pharmacology and efficacy of liposome-entrapped albendazole in experimental secondary alveolar echinococcosis and effect of coadministration with cimetidine. Parasitol. 113, 111–121.

Zhou, Y., Du, J., Wang, L., Wang, Y. 2016. State of the art of nanocrystals technology for delivery of poorly soluble drugs. J. Nanopart. Res. 18(9), 257.

Legends to figures:

Fig. 1. Scanning and transmission electron microscopy (SEM and TEM) of *E. multilocularis* cysts recovered from mice treated with albendazole nanocrystals (ABZ-NCs) or albendazole (ABZ) suspension during the chemoprophylactic study. Both formulations were given at 5 mg/kg of ABZ. Representative images of metacestodes recovered from control group: (a) Germinal layer with different cell types (SEM 400×); (b) Laminar and germinal layers showing normal appearence (TEM 12000×). Representative images of metacestodes recovered from ABZ suspension group: (c) Altered germinal layer (SEM 400×); (d) Damaged internal tissue with reduction in microtriches number (TEM 10000×). Representative images of metacestodes recovered from ABZ-NC group: (e) Only few cells are observed in the germinal layer (SEM 400×). (f) Internal tissue strongly altered. See the presence of electron-dense structures in michocondria (white arrows), several vacuoles and the depletion of glycogen storage cells (TEM 10000×). Te: tegument; GL: germinal layer; LL: laminar layer; gly: glycogen storage cells; mu: muscle cells; v; vacuoles; black arrows: microtriches.

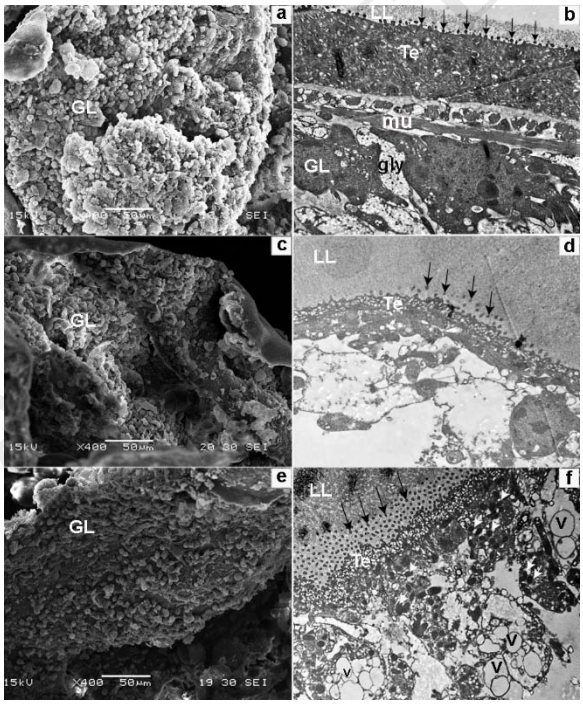


Fig. 2. (A) Protoscoleces viability (%, mean±SD) recovered from control and treated mice 24 h post-infection with *E. multilocularis*. (B) SEM image of protoscoleces isolated from control mice or mice treated with albendazole nanocrystals (ABZ-NCs) or albendazole (ABZ) suspension. (a) Control protoscolex (750×). (b) Magnification of scolex region of a control protoscolex (1100×). (c) Protoscolex recovered from mice treated with ABZ suspension. Note the contraction of the soma region and rostelar disorganization (800×). (d) Protoscolex isolated from mice treated with ABZ-NCs. Loss of morphology is evident (800×). (e) Scolex region of protoscolex isolated from ABZ-NC group. Observe the rostelar disorganization and loss of microtriches (1500×). sr: soma region; rr: rostelar region; s: sucker.

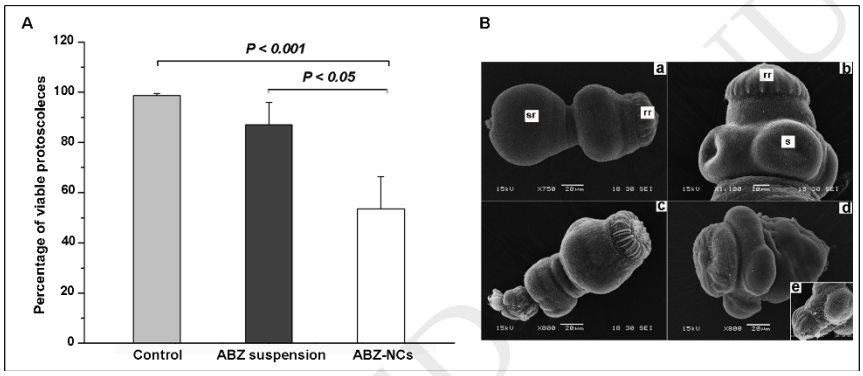


Fig. 3. Scanning and transmission electron microscopy (SEM and TEM) of *E. multilocularis* cysts recovered from mice treated with albendazole nanocrystals (ABZ-NCs) or albendazole (ABZ) suspension during the clinical efficacy study. Both formulations were given at 5 mg/kg of ABZ. Representative images of metacestodes recovered from control group: (a) Germinal layer with different cell types (SEM 800×). (b) TEM image (10000×). Representative images of metacestodes recovered from ABZ suspension group: (c) Diminishing in germinal cell number can be observed (SEM 800×). (d) Metacestode with altered internal tissue and reduction in microtriches number (TEM 10000×). Representative images of metacestodes recovered from ABZ-NC group: (e) Germinal layer strongly altered with presence of cellular debris (SEM

800×). (f) Internal tissue completely distorted and marked microtriches reduction (TEM 10000×). GL: germinal layer; LL: laminar layer; Te: tegument; arrows: microtriches.

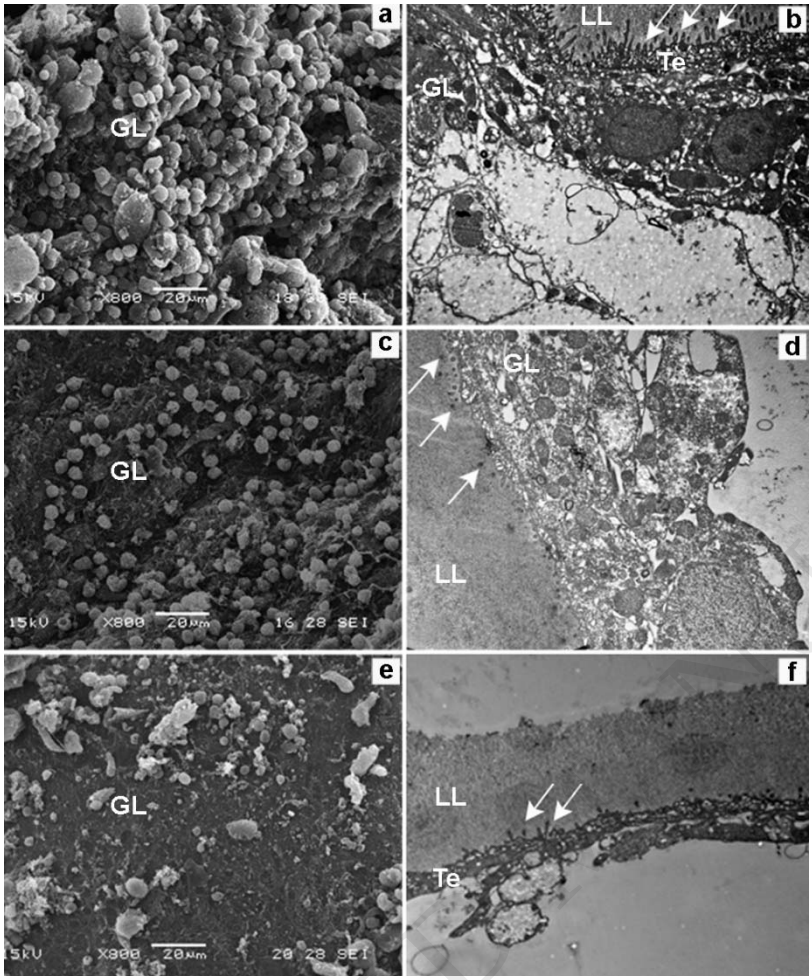


Fig. 4. (A) Protoscoleces viability (%, mean±SD) recovered from control mice and mice treated with albendazole nanocrystals (ABZ-NCs) or albendazole (ABZ) suspension 7 weeks post-infection with *E. multilocularis*. B) SEM image of protoscoleces isolated from control and treated mice with ABZ-NCs (5mg/kg) or ABZ suspension (5mg/kg). (a) Control protoscoleces budding from cyst germinal layer (arrows, 230×). (b) Evaginated control protoscolex (650×). (c) Protoscolex isolated from ABZ suspension group. Note the loss of hooks (650×). (d) Protoscolex isolated from ABZ-NC group. Loss of the typical morphology can be observed (650×).

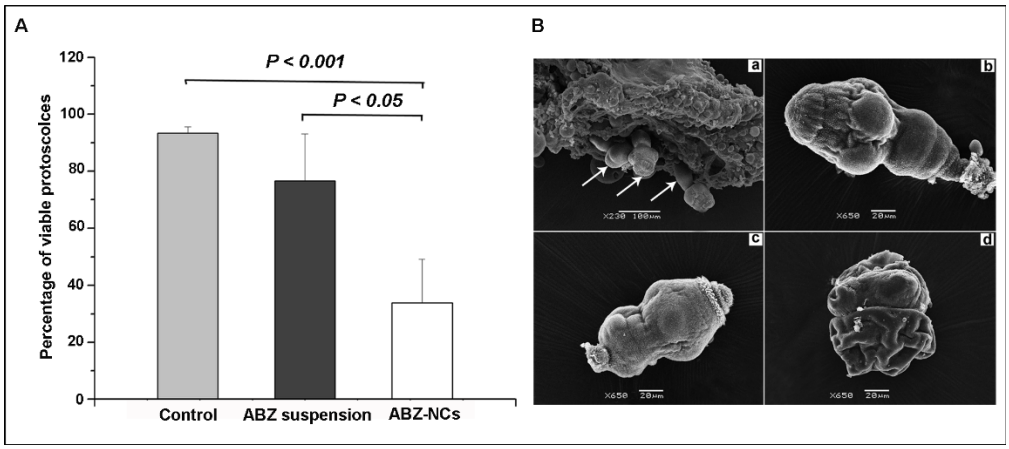


Table 1. Chemoprophylactic efficacy study. Mean (±SD) weights (g) of *E. multilocularis* cysts recovered at 11 weeks post-infection from intraperitoneally infected mice from thecontrol, albendazole nanocrystal (ABZ-NC) and albendazole (ABZ) suspension groups. Drug administration started one day post-infection. Treatments were given at 5 mg/kg of ABZ, every 24 h over a period of 30 days.

	Chemoprophylactic efficacy study	
	Wet weight (g) of cyst Mean±SD	**% of efficacy**
Control group	13.15 ± 4.51	
Blanck-NC group	15.05 ± 5.31	
ABZ suspension group	16.04 ± 3.89	0
ABZ-NC group	7.84 ± 2.15[a b]	50.89

[a] $P < 0.01$, statistically significant differences between ABZ-NC group vs. control groups.
[b] $P < 0.01$, statistically significant differences between ABZ-NC group vs. ABZ suspension groups.

Table 2. Clinical efficacy study. Mean (±SD) weights (g) of *E. multilocularis* cysts recovered at 11 weeks post-infection from intraperitoneally infected mice from the control, albendazole nanocrystal (ABZ-NC) and albendazole (ABZ) suspension groups. Drug administration started 7 weeks post-infection. Treatments were given at 5 mg/kg of ABZ, every 24 h over a period of 30 days.

	Clinical efficacy study	
	Wet weight (g) of cyst Mean±SD	**% of efficacy**

Control group	9.07 ± 2.46	
Blanck-NC group	11.05 ± 5.31	
ABZ suspension group	4.9 ± 2.2	45.95
ABZ-NC group	2.17 ± 1.24 [a]	76.07

[a] $P < 0.01$, statistically significant differences between ABZ-NC group vs. control groups.