In vitro efficacy of albendazole-loaded β -cyclodextrin against protoscoleces of *Echinococcus granulosus sensu stricto*

Nayer Mehdizad Bakhtiar

Tabriz University of Medical Sciences Faculty of Medicine

Abolfazl Akbarzadeh

Tabriz Medical University: Tabriz University of Medical Sciences

Ehsan Ahmadpour

Tabriz University of Medical Sciences

Mahmoud Mahami-Oskouei

Tabriz University of Medical Sciences

Adriano Casulli

Italian Institute for Genomic Medicine

Roghayeh Norouzi

Tabriz University: University of Tabriz

Milad Asadi

Tabriz University of Medical Sciences

Mina Ebrahimi

Tabriz University of Medical Sciences

Nahideh Asadi

Tabriz Medical University: Tabriz University of Medical Sciences

Sonia M. Rodrigues Oliveira

University of Aveiro: Universidade de Aveiro

Maria de Lourdes Pereira

University of Aveiro: Universidade de Aveiro

Adel Spotin (✉ adelespotin@gmail.com)

Tabriz University of Medical Sciences <https://orcid.org/0000-0002-3009-9537>

Research Article

Keywords: *Echinococcus granulosus*, Protoscoleces, β -cyclodextrins, Albendazole, Apoptosis, Arginase, Thioredoxin peroxidase.

Posted Date: February 28th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1362819/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Abstract

Background: Cystic echinococcosis (CE), a widespread helminthic disease caused by the larval stage of the dog tapeworm *Echinococcus granulosus* is represented as a public health concern in humans. The therapeutic failure of albendazole (ABZ) against CE depends on its low aqueous solubility and consequently its erratic bioavailability in plasma. Serious adverse effects have been also observed following the long-term use of ABZ *in vivo*.

Methods: We evaluated the apoptotic effects of ABZ-loaded β -cyclodextrin (ABZ- β -CD) against protoscoleces (PSCs) *versus* ABZ alone. After 15 h of exposure time, Caspase-3 enzymatic activity was determined by fluorometric assay in PSCs treated with ABZ and ABZ- β -CD. To assess the treatment efficacy of ABZ- β -CD against PSCs, mRNA expression of Arginase (*EgArg*) and Thioredoxin peroxidase (*EgTPx*) were quantified by Real-time PCR.

Results: A significant scolical activity of ABZ was observed only at a concentration of 800 $\mu\text{g/mL}$ (100% PSCs mortality rate after 4 days of exposure), while the 200 and 400 $\mu\text{g/mL}$ ABZ reached 100% PSCs mortality rate after 9 sequential days. The 400 $\mu\text{g/mL}$ ABZ- β -CD had 100% scolical rate after 5 days of exposure. Morphological alterations using scanning electron microscopy in treated PSCs revealed that ABZ and ABZ- β -CD induced higher Caspase-3 activity than their controls, indicating a more potent apoptotic outcome on the PSCs. Also, we showed that the ABZ- β -CD can down-regulate the mRNA expression of *EgArg* and *EgTPx*, indicating more potent interference against growth and antioxidant properties of PSCs.

Conclusions: These data provide new insights into the nanostructured β -CD carriers of ABZ appear as a promising candidate to improve the treatment of CE *in vivo* models.

Background

Cystic echinococcosis (CE) is a neglected helminthic infection with significant impacts on human health caused by the larval stages of *Echinococcus granulosus sensu lato* (s.l.) [1, 2]. Canids harboring adult worms of *E. granulosus* s.l. excrete Echinococcal eggs into the environment, where humans and animals may be infected via the ingestion of the eggs. Once ingested, the oncospheres in the eggs hatch, penetrate the intestinal wall of the host and migrate through the blood and lymphatic vessels to the primary target organs such as the liver and secondarily the lungs where they eventually develop into fertile hydatid cysts [3, 4].

Depending on the location, size, number and staging of the human CE, there are four treatment options: surgery, percutaneous treatments, pharmacological therapy with benzimidazoles (BMZs) and “watch and wait” [5]. In most endemic countries, surgery is the main practiced interventional option, however, the antiparasitic drug treatment is necessary for inoperable cases or in combination with surgery and percutaneous treatments to prevent recurrence of secondary cysts [6]. Among BMZs, albendazole (ABZ) is the most effective therapeutic option for the treatment of CE. Nevertheless, ABZ exerts a parasitostatic

rather than a parasitocidal effect [7–9]. Therapeutic failure with ABZ has been widely reported due to its low aqueous solubility and consequently its erratic bioavailability in both plasma and hydatid cyst fluid [7, 10]. Moreover, serious adverse effects such as liver toxicity, leukopenia, alopecia, thrombocytopenia and teratogenic effects have been observed following the long-term use of ABZ [11–13]. To overcome this therapeutic gap, several approaches have been developed to improve the bioavailability of poor soluble BMZs in animal *in vivo* models. Among these, an affordable synthetic approach based on the presence of the acidic NH group in the BMZs nucleus has been shown with the aim to get patented new highly water-soluble sodium salts of Ricobendazole (ABZSO) and its enantiomers [14]. Moreover, nanomaterials as delivery systems of BMZs have been recently evaluated against hydatid cysts, and in spite of their limitations, promising scolicial and cysticidal outcomes have shown to improve the treatment of CE [15, 16]. Polymeric combinations originating from synthetics or natural derivatives have been identified as the most effective nanoparticles (NPs) due to their high encapsulation capability, hydrophilic radicals and controlled release of drugs in the treatment of CE [7, 15]. Cyclodextrin (CD) has been widely used as biodegradable carriers with a hydrophilic outer surface and lipophilic central cavity for drug delivery to increase the solubility of poorly water-soluble drugs. There are three common types of CD including α , β and γ which consist of six, seven and eight glucose subunits, respectively [17, 18]. Among them, β -CD is ideal for encapsulation because of its perfect cavity size and efficient drug loading capacity [19–22]. To the best of our knowledge, there is no *in vitro* study on parasitocidal effect of ABZ loaded with β -CD against PSCs of *E. granulosus sensu stricto* (s.s.).

Naturally, CE resides in the semi-aerobic environment of the intermediate hosts, consequently hydatid cysts are continuously exposed to reactive oxygen species (ROS) [23]. Due to this fact, hydatid cysts of *E. granulosus* have developed effective defense systems of enzymatic antioxidants to protect themselves against potentially damaging effects of ROS [24]. Thioredoxin peroxidase (TPx) is one of the peroxiredoxin (Prx) gene superfamily, which can play a key role in the antioxidant defense system of *E. granulosus* [23, 25]. However, the nano-drug effects on the regulatory function of *E. granulosus* TPx (*EgTPx*) have not yet been fully understood. On the one hand, evidence shows that the *E. granulosus* arginine (*EgArg*) and inducible nitric oxide synthase (iNOS) compete for L-arginine substrate and are involved in parasite protection or host defense, respectively [26]. Arg converts L-arginine to L-ornithine and ultimately leads to the synthesis of polyamines that participate in important cellular activities including growth, differentiation and proliferation. Furthermore, L-ornithine may act as nitric oxide synthase (NOS) substrate, which causes apoptosis of parasite by producing NO [27, 28]. Recent studies have been indicated that Arg activity is expressed in mammalian cells infected with *E. granulosus* [26, 28]. Further studies have also revealed that the drug-induced apoptosis can be triggered by Caspase-3 enzymatic activity in the protoscoleces (PSCs) [29–31].

Some studies have been shown that apoptosis can be induced following activation of arginase and TPx in the interaction of cancer cells/bacterial pathogenesis with therapeutic interventions [32, 33]. Due to this fact, we hypothesized whether ABZ and ABZ-loaded β -cyclodextrin (ABZ- β -CD) can effectively reduce the growth and antioxidant properties of *E. granulosus*.

The main aim of this study was to evaluate and compare the scolical and apoptotic effects of ABZ- β -CD *versus* ABZ *in vitro*. In addition, to assess the treatment efficacy of ABZ- β -CD against PSCs of *E. granulosus* s.s., the mRNA expression levels of *EgArg* and *EgTPx* were quantified by Real-Time PCR tool.

Methods

Preparation of ABZ-loaded β -CD

ABZ and β -CD were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 100 mg of β -CD were dissolved in 16 mL deionized water; then 30 mg of ABZ was solubilized in 1,000 μ L dimethyl sulfoxide (DMSO). The solutions were mixed and placed on the stirrer at 400 rpm for 24 h to evaporate the DMSO without a cap. Afterward, it was centrifuged at 10,000 rpm for 5 min and supernatant was collected by freeze-drying.

Encapsulation and characterization of ABZ- β -CD

The morphological structure of the ABZ- β -CD was detected by scanning electron microscopy (SEM). One mg of the freeze-dried sample was re-dispersed in 1 mL DMSO and mounted on aluminum foil. After drying, the sample was coated with gold particles and imaged by SEM. The ABZ- β -CD was validated by fourier transform infrared (FTIR) spectra. The FTIR spectrum for blank and drug-loaded NPs was obtained using a neat film cast of the chloroform copolymer solution between KBr discs. The zeta (ζ) potential and particle size distribution were performed by Zetasizer (Malvern Instruments Ltd, Malvern, UK). The formulation consisting of ABZ- β -CD was prepared using the freeze-drying method of [34]. One mg of ABZ- β -CD was dissolved in 100 mL DMSO and placed in a shaker for 24 h at room temperature. Then, the solution was centrifuged at 12,000 rpm and the supernatant was collected for the calculations. ABZ concentration in the filtrate was measured by UV spectrophotometry at 291 nm (Boeco S-26 spectrometer, Hamburg, Germany).

Protoscoleces collection, culturing and genotyping

Fertile hydatid cysts (containing PSCs) were obtained from liver of infected sheep slaughtered in Northwest Iran (Fig. 1A). The hydatid cyst fluid (HCF) was aseptically aspirated and transferred to 50 mL sterilized falcon tubes and then fixed to settle the PSCs. PSCs were washed three times with phosphate buffered saline (pH: 7.2). The viability of the PSCs was authenticated by motility of flame cells and staining with 0.1% eosin (1 g of eosin powder in 1000 ml distilled water) under a light microscope. Finally, the RPMI-1640 medium (Gibco™, Thermo Fisher Scientific) containing 1% double-antibiotics (100 U/L penicillin plus 100 mg/L streptomycin) were added to the living PSCs with more than 95% viability and incubated in 5% CO₂ at 37°C for further investigations. To identify the genotype of *E. granulosus*, genomic DNA of PSCs was extracted with a commercial kit (DNG-plus™ solution, Iran). The extracted DNA was amplified by single-round PCR targeting of Cytochrome C oxidase 1 (*Cox1*; 444 bp amplification) [35]. Amplicons of *Echinococcus* were sequenced (Codon, Tehran, Iran), edited and confirmed by a phylogenetic tree (MEGA5.0 software).

Scolicidal assay

In the current study, β -CD and ABZ were dissolved in a final concentration of 0.1% DMSO (0.1 mL of DMSO + 99.9 mL of water). PSCs treated with DMSO and β -CD were used as negative controls for ABZ and ABZ- β -CD, respectively. Drug concentrations of 100, 200, 400 and 800 μ g/mL were tested for 9 sequential days to reach equimolar ABZ concentrations between ABZ- β -CD and ABZ alone. To prepare the concentrations of 100, 200 and 400 μ g/mL, the 125, 250 and 500 μ L of ABZ and ABZ- β -CD were separately dissolved in 875 μ L, 750 μ L and 500 μ L of RPMI-1640, respectively. As well, to prepare 800 μ g/mL, 1000 μ L of ABZ and 1000 μ L of ABZ- β -CD were directly treated with PSCs. Then 10^3 PSCs per well were plated in a 24-well plate containing RPMI-1640 and incubated at 37°C in 5% CO₂. PSCs treated with different concentrations of ABZ and ABZ- β -CD groups, were daily (for 9 days) observed under a light microscope for the evaluation of their viability.

The viability of PSCs was checked by using 0.1% aqueous eosin stain and by observing flame cell activity. Nonviable (dead) PSCs were absorbed eosin, but alive ones remained unstained (colorless) (Fig. 1B-1C). The percentages of dead PSC were estimated by counting a minimum of 300 PSCs per drug concentration.

Electron microscopy

Ultrastructural alterations were evaluated using SEM. The method previously described by Naseri et al. (2016) was followed. The collected PSCs were washed in 100 mM phosphate buffer (pH: 7.2) and fixed in 3% glutaraldehyde for 12 h at room temperature. The specimens were dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%) and were immersed in hexamethyl disilazane for 5 min, 1 h, and then, overnight. The samples were sputter-coated with gold and inspected on a JEOL JSM-6460 LV SEM operating at 15 kV.

Detection of enzymatic activity of Caspase-3

After 15 h of exposure to ABZ and ABZ- β -CD-treated PSCs (10^3), the Caspase-3 activity for concentrations of 400 and 800 μ g/mL and control groups was characterized by the Caspase-3/CPP32 Fluorometric Assay Kit [36]. Caspase-3 activity assays were repeated at least three times in triplicate.

Primer designing, extraction of total RNA, synthesis of complementary DNA (cDNA and quantitative real-time PCR (qPCR)

In order to assess the treatment efficacy of ABZ and ABZ- β -CD against cultured PSCs, the specific primers of *EgArg* and *EgTPx* were designed by an Oligo Analyzer based on accession numbers of XM-024496307.1 (*EgArg*) and EU696759 (*EgTPx*). After 6 h of exposure time, harvesting of total RNA from PSCs treated with ABZ and ABZ- β -CD was carried out based on the TRIzol lysing reagent. The quality and quantity of isolated RNA were evaluated by a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). cDNAs were synthesized using total RNA harvested from PSCs treated with ABZ and ABZ- β -CD and controls. For quantitative assessment of *EgArg* and *EgTPx*, qPCR was done using

QuantiFast SYBR Green Takara PCR kit. Oligonucleotide primers and their fragment sizes are listed in Table 1. All qPCR reactions were repeated in duplicate with employing elongation factor-1 alpha (*EF-1α*) as the housekeeping internal control. To analyze parasite-derived mRNAs, the relative fold change among groups and controls was determined and normalized to *EF-1α*. The relative fold gene expression of Caspase-3, *EgArg* and *EgTPx* were calculated using Ct ($2^{-\Delta\Delta C_t}$) formula.

Table 1
The sequence of primers designed for the quantitative real-time PCR (qPCR).

Primer	Sequence (s)	(Base pair)	Accession number
EF-1α (<i>Echinococcus granulosus</i> housekeeping)	F:5'-TTTGAGAAAGAGGCGGCTGAGGTG-3' F:5'-TAATAAAGTCACGATGACCGGGCG-3'	208	AB306934
Arginase (<i>Echinococcus granulosus</i>)	F:5'- GCAGAACGTTTGAAGGTAA-3' F:5'-TATATCGGCGTGAGTGTCCA-3'	198	XM_024496307.1
Thioredoxin peroxidase (<i>Echinococcus granulosus</i>)	F:5'- AAGTTTCACGTGCAAGGCTCT-3' F:5'-GGACAGACGAAGGYGCTAGG-3'	186	EU696759

Statistical analysis

Differences between the test and control groups for nominal variables were analyzed using the Mann–Whitney U-test and one-way analysis of variance (ANOVA). *P* value < 0.05 was considered significant. In addition, graphs were constructed using GraphPad Prism software version 5.0.

Results

FTIR spectroscopy, zeta potential, particle size and SEM analyze

FTIR spectroscopy was employed to demonstrate the structure of ABZ (Supplementary Fig. 2A), β-CD and ABZ-β-CD (Supplementary Fig. 2B and 2C). The FTIR spectra of these polymers showed a broad absorption at 3,334 cm – 1 representing the presence of –OH radicals. Also, absorption at 2,920 cm – 1 indicated the presence of –CH radicals and absorption band at 1,030 – 1,092 cm – 1 confirms the formation of C–O–C ether bond. Absorption at 1,635 cm – 1 showed the presence of C = O radicals and absorption at 2,162 cm – 1 demonstrating the presence of C = N radicals. Zeta potential determination of ABZ-β-CD is also shown in Fig. 3A (Supplementary Fig). The SEM micrograph of ABZ-β-CD layered with

gold particles is shown in Fig. 3B (Supplementary Fig). The average diameter calculated by laser particle size measurement was 12.09 nm to 14.21 nm (Supplementary Fig. 3B). As seen in Fig. 3B, the ABZ-loaded β -CD were well dispersed in the solution. The encapsulation efficiency (entrapment efficiency) of ABZ- β -CD was 97%, as calculated by high-performance liquid chromatography and Sephadex® gel filtration.

Scolicidal effects of ABZ and ABZ- β -CD

Scolicidal effects at various concentrations (100, 200, 400 and 800 μ g/mL) of ABZ and ABZ- β -CD were investigated for 9 sequential days. A significant scolicidal activity of ABZ was observed only at a concentration of 800 μ g/mL (100% PSCs mortality rate after 5 days of exposure), while the 200 and 400 μ g/mL ABZ reached 100% PSCs mortality rate after 9 days of exposure (Fig. 4A). As well, 100 μ g/mL ABZ reached 90% mortality rate after 9 days (Fig. 4A).

On the other hand, 800 and 400 μ g/mL ABZ- β -CD had 100% PSCs mortality rate after 5 and 6 days of exposure, whereas a concentration of 200 μ g/mL ABZ- β -CD had 100% scolicidal rate on day 9 (Fig. 4B). 100 μ g/mL ABZ- β -CD reached 88% scolicidal rate after 9 days (Fig. 4B). Although 800, 200 and 100 μ g/mL ABZ- β -CD had similar PSCs mortality rates compared to ABZ, 400 μ g/mL ABZ- β -CD showed a higher scolicidal rate (100%) than ABZ alone (35%) (Fig. 4A and 4B).

Morphological alterations of PSCs treated with ABZ- β -CD and phylogenetic tree

Morphological changes in PSCs treated with β -CD, ABZ- β -CD, DMSO and ABZ were evaluated after 24 h of exposure time. No morphological alterations were observed in PSCs treated with β -CD and PSCs treated with the 0.1% DMSO during the treatment times (Fig. 5A and Fig. 5C); whereas morphological alterations in PSCs treated with ABZ- β -CD showed a significant disrupted surface compared to ABZ alone (Fig. 5B and Fig. 5D). Based on sequencing and phylogenetic analyses of mitochondrial *Cox1* gene, the G1 genotype of *E. granulosus* s.s. was identified as causative species.

Evaluation of Caspase-3 enzymatic activity

The Caspase-3 activity after 15 h of exposure to PSCs treated with 400 μ g/mL ABZ and 400 μ g/mL ABZ- β -CD was higher compared to their control groups (Fig. 6). Statistically, ABZ at 400 μ g/mL induced a higher Caspase-3 activity than 400 μ g/mL ABZ- β -CD (*P* = 0.00012). No significant apoptotic difference observed between 800 μ g/mL ABZ/800 μ g/mL ABZ- β -CD and their controls (Fig. 6).

mRNA expression levels of *EgArg* and *EgTPx*

We showed that the treatment with 200, 400 and 800 μ g/mL ABZ- β -CD can significantly down-regulate the mRNA expression of *EgArg* compared to ABZ alone in the respective concentrations (Fig. 7A), however, there was no significant difference between the treatment group of 200 μ g/mL ABZ and the

control. Our results show that the 400 µg/mL ABZ-β-CD can significantly down-regulate the *EgTPx* mRNA expression in treated PSCs compared with the control (P : 0.00043) (Fig. 7B), while an up-regulation of about 1.5-fold change was observed in PSCs treated with 800 µg/mL ABZ-β-CD compared to 400 µg/mL ABZ-β-CD.

Discussion

In the present study, aiming to increase the treatment efficacy of ABZ against PSCs of *E. granulosus* s.s., we compared the *in vitro* cestocidal and apoptotic properties of ABZ *versus* ABZ-β-CD. Limitations observed in the current ABZ formulation, such as the parasitostatic effect and possible drug resistance to BMZs have lead to the importance of nanomedicine for developing fine-tuned formulations including solubility and stability of ABZ as long-term delivery systems [7, 15].

In this study, concentrations of 100, 200 and 800 µg/mL ABZ-β-CD had similar PSCs mortality rates compared to ABZ alone, while 400 µg/mL ABZ-β-CD showed a higher scolical rate (100%: on the sixth day of exposure) than ABZ (35%; on the ninth day of exposure).

The presence of hydrophilic radicals (hydroxyl groups ~OH; $n = 27$) in the β-CD structure can enhance the solubility of ABZ, increasing the mortality ability against PSCs compared to ABZ alone. Also, ABZ-β-CD may effectively cause degenerative alterations in the surface of PSCs by binding to the colchicine-sensitive site of β-tubulin, consequently inhibiting its polymerization into microtubules [37].

The improved bioavailability and anthelmintic activity of ABZ using β-CD and their synthetic derivatives have been investigated in pharmaceutical research [38–40]. Previous studies on the use of CD-based flubendazole formulations (FLBZ) in infected mice have shown that CD improves the pharmaceutical behavior of FLBZ, leading to increased exposure to hydatid cysts and better clinical efficacy in CE [20–22, 41]. Other studies have indicated that the hydroxypropyl-β-CD can improve the effectiveness of ABZ against encapsulated larvae of *Trichinella spiralis* in a murine model [20, 42–44].

Similar investigations have also employed novel nanocompounds as CE treatment approaches. In a recent study, it has been shown that ABZ-loaded solid lipid NPs after 5 days have higher scolical effects at both concentrations (250 and 500 µg/mL) than ABZ alone [45]. An *in vitro* study showed that ABZ sulfoxide (ABZSO) and ABZSO-loaded PLGA-PEG have 100% mortality rate against PSCs at low concentrations of 150 and 200 µg/mL, six times faster than that of ABZSO alone [29]. Similar studies have reported that ABZ-loaded lipid nanocapsules are therapeutically more effective than ABZ suspension and have shown more ultrastructural modifications in CE experimentally infected mice [46, 47].

In this study, PSCs treated with 400 µg/mL ABZ and 400 µg/mL ABZ-β-CD showed higher enzymatic activity of Caspase-3 than controls, indicating a more potent apoptotic outcome on the PSCs. On the one hand, the 400 µg/mL ABZ-β-CD had 100% PSCs mortality rate compared to 400 µg/mL ABZ alone (40% scolical rate), however, ABZ (400 µg/mL) induced a higher apoptotic activity than ABZ-β-CD (400

µg/mL) after 15 h exposure time. This probably indicates that PSCs treated with ABZ-β-CD may acquire resistance to apoptosis by the expression of anti-apoptotic proteins or down-regulation of pro-apoptotic proteins, therefore a measurement of Caspase-3 activity in PSCs treated with 400 µg/mL ABZ-β-CD in various exposure times is needed to verify this reverse function.

In agreement with our results, various studies on drug-induced apoptosis have confirmed an increased induction of apoptosis of PSCs through Caspase-3-dependent pathway [29, 36, 48–50]. Evaluation of apoptotic activity in PSCs treated with ABZSO and ABZSO-loaded PLGA-PEG indicated that the mRNA expression level of Caspase-3 was higher in both drugs than in the control group [29]. In this study, for the first time to demonstrate the treatment efficacy of ABZ-β-CD against co-cultured PSCs, the specific primers of *EgArg* (as growth candidate gene) and *EgTPx* (as antioxidant candidate gene) were designed and measured. PSCs treated with ABZ and ABZ-β-CD showed that the mRNA expression of *EgArg* was lower than that in controls, indicating more potent interference against the growing of PSCs. However, ABZ-β-CD showed a 2.5-fold change decrease in mRNA expression of *EgArg* compared to ABZ alone.

It has recently been shown that ABZ-induced ROS can inhibit protozoan *Giardia duodenalis* growth through mechanisms of oxidative stress and apoptosis [51]. On the other hand, *Dicrocoelium dendriticum* treated with ABZ can increase the activity of antioxidant enzymes in response to toxic effects [52]. The imbalance between ROS production and antioxidant defense leads to a condition called oxidative stress that causes cellular dysfunction and cell death [53]. It has been previously shown that the laminated layer of *E. granulosus* can induce the Arg pathway to overcome the host immune responses [26]. Hanedan et al. (2015) indicated that an increased Arg activity is an important factor in the development of CE-related pulmonary fibrosis in cows [28].

Another study has determined that parasitic Arg may use host arginine to reduce its availability for iNOS activity and thereby decrease NO production [27]. In this study, in order to confirm the theoretical role of Arg, it would be suggested to assess with measuring protein or enzymatic activity level.

Our results indicate that the *EgTPx* expression in PSCs treated with 400 µg/mL ABZ-β-CD was significantly reduced compared to the control. Down-regulation of *EgTPx* significantly can decrease the ability of fertile cysts to anti oxidative stress and consequently impair the growth of CE cysts [23]. In this regard, it has been shown that *EgTPx* is also particularly over-expressed in calcareous corpuscles of PSCs and can be released rapidly to protect *E. granulosus* against oxidative damage [25].

The lower expression of Arginase or Thioredoxin may be explained by apoptosis of PSCs due to the treatment. To verify the hypothesis for this mode of action, experiments with specific inhibitors or RNAi-mediated knock-downs of *EgArg* and *EgTPx* are needed that show that the down-regulation of these genes increases oxidative stress and Caspase-3 activity.

Conclusions

In the present study, a significant difference was observed in the scolical effects, apoptosis intensity and treatment efficacy between PSCs treated with 400 µg/mL ABZ and PSCs treated with 400 µg/mL ABZ-β-CD. *In vivo* studies of ABZ-β-CD and further similar investigations are still needed to develop more effective ABZ nano-compounds and provide the new insights into understanding of reactions between ABZ-β-CD and PSCs.

Abbreviations

BMZs
Benzimidazoles
ABZ
Albendazole
CD
Cyclodextrin
EgArg
E. granulosus arginine
EgTPx
E. granulosus Thioredoxin peroxidase
PSCs
Protoscoleces
iNOS
Nitric oxide synthase
DMSO
Dimethyl sulfoxide
FTIR
Fourier transform infrared
HCF
Hydatid cyst fluid.

Declarations

Acknowledgments

This is a report of a database from the thesis of Miss Nayer Mehdizad Bakhtiar registered in Tabriz University of Medical Sciences. This work was also partially supported by ERANet-LAC 2nd Joint Call (<http://www.eranet-lac.eu>) and the Italian Ministry of Health-NDTND project.

Funding

This study was financially supported by Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran (Grant no. 59631).

Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Authors' contributions

NMB&AS&NA: carried out the molecular genetic studies and have been involved in drafting the manuscript. MMO&EH&AK&RN&MLP: participated in the design of the study, contributed to data collection and helped to draft the manuscript. AC&MA&ME&SMRO: performed the statistical analysis and have been involved in critically revising the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

IR.TBZMED.VCR.REC.1397.050.

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no conflict of interests.

References

1. Casulli A, Siles-Lucas M, Tamarozzi F. *Echinococcus granulosus* sensu lato. Trends Parasitol. 2019.
2. Eckert J, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin Microbiol Rev. 2004;17(1):107–35.
3. McManus DP, Zhang W, Li J, Bartley PB. Echinococcosis Lancet. 2003;362(9392):1295–304.
4. Hajizadeh M, Ahmadpour E, Sadat ATE, Spotin A. Hydatidosis as a cause of acute appendicitis: A case report. Asian Pac J Trop Dis. 2013;3(1):71–3.
5. Brunetti E, Kern P, Vuitton DA. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 2010;114(1):1–16.
6. Higueta NIA, Brunetti E, McCloskey C. Cystic echinococcosis. J Clin Microbiol. 2016;54(3):518–23.
7. Siles-Lucas M, Casulli A, Cirilli R, Carmena D. Progress in the pharmacological treatment of human cystic and alveolar echinococcosis: Compounds and therapeutic targets. PLoS Negl Trop Dis. 2018;12(4):e0006422.
8. Kohansal MH, Nourian A, Rahimi MT, Daryani A, Spotin A, Ahmadpour E. Natural products applied against hydatid cyst protoscolices: a review of past to present. Acta Trop. 2017;176:385–94.
9. Lundström-Stadelmann B, Rufener R, Ritler D, Zurbriggen R, Hemphill A. The importance of being parasitocidal... an update on drug development for the treatment of alveolar echinococcosis. Food

Waterborne Parasitol. 2019;15:e00040.

10. Lötsch F, Naderer J, Skuhala T, Groger M, Auer H, Kaczirek K, Waneck F, Ramharther M. Intra-cystic concentrations of albendazole-sulphoxide in human cystic echinococcosis: a systematic review and analysis of individual patient data. *Parasitol Res.* 2016;115(8):2995–3001.
11. Hemphill A, Stadelmann B, Rufener R, Spiliotis M, Boubaker G, Müller J, Müller N, Gorgas D, Gottstein B: Treatment of echinococcosis: albendazole and mebendazole–what else? *Parasite* 2014; 21.
12. Horton J. Albendazole: a review of anthelmintic efficacy and safety in humans. *Parasitology.* 2000;121(S1):113–32.
13. John H. Albendazole for the treatment of echinococcosis. *Fundam Clin Pharmacol.* 2003;17(2):205–12.
14. Cirilli R, Guglielmi P, Formica FR, Casulli A, Carradori S. The sodium salt of the enantiomers of ricobendazole: Preparation, solubility and chiroptical properties. *J Pharm Biomed Anal.* 2017;139:1–7.
15. Bakhtiar NM, Akbarzadeh A, Casulli A, Mahami-Oskouei M, Ahmadpour E, Nami S, Rostami A, Spotin A. Therapeutic efficacy of nanocompounds in the treatment of cystic and alveolar echinococcoses: challenges and future prospects. *Parasitol Res.* 2019;118(9):2455–66.
16. Ritler D, Rufener R, Sager H, Bouvier J, Hemphill A, Lundström-Stadelmann B. Development of a movement-based in vitro screening assay for the identification of new anti-cestodal compounds. *PLoS Negl Trop Dis.* 2017;11(5):e0005618.
17. Pacheco P, Rodrigues L, Ferreira J, Gomes A, Veríssimo C, Louvandini H, Costa R, Katiki L. Inclusion complex and nanoclusters of cyclodextrin to increase the solubility and efficacy of albendazole. *Parasitol Res.* 2018;117(3):705–12.
18. Kalepu S, Nekkanti V. Insoluble drug delivery strategies: review of recent advances and business prospects. *Acta Pharm Sinica B.* 2015;5(5):442–53.
19. Gidwani B, Vyas A: A comprehensive review on cyclodextrin-based carriers for delivery of chemotherapeutic cytotoxic anticancer drugs. *Biomed Res Int.* 2015; 2015.
20. Ceballos L, Elissondo C, Bruni SS, Denegri G, Lanusse C, Alvarez L. Comparative performances of flubendazole and albendazole in cystic echinococcosis: ex vivo activity, plasma/cyst disposition, and efficacy in infected mice. *Antimicrob Agents Chemothe.* 2011;55(12):5861–7.
21. Ceballos L, Elissondo C, Bruni SS, Confalonieri A, Denegri G, Alvarez L, Lanusse C. Chemoprophylactic activity of flubendazole in cystic echinococcosis. *Chemotherapy.* 2010;56(5):386.
22. Ceballos L, Elissondo M, Bruni SS, Denegri G, Alvarez L, Lanusse C. Flubendazole in cystic echinococcosis therapy: pharmaco-parasitological evaluation in mice. *Parasitol Int.* 2009;58(4):354–8.
23. Wang H, Li J, Zhang C, Guo B, Wei Q, Li L, Yang N, McManus DP, Gao X, Zhang W. *Echinococcus granulosus* sensu stricto: silencing of thioredoxin peroxidase impairs the differentiation of protoscoleces into metacestodes. *Parasite.* 2018; 25.

24. McGonigle S, Dalton J, James E. Peroxidoxins: a new antioxidant family. *Parasitol Today*. 1998;14(4):139–45.
25. Li J, Zhang W-B, Loukas A, Lin R-Y, Ito A, Zhang L-H, Jones M, McManus DP. Functional expression and characterization of *Echinococcus granulosus* thioredoxin peroxidase suggests a role in protection against oxidative damage. *Gene*. 2004;326:157–65.
26. Amri M, Touil-Boukoffa C. A protective effect of the laminated layer on *Echinococcus granulosus* survival dependent on upregulation of host arginase. *Acta Trop*. 2015;149:186–94.
27. Badirzadeh A, Taheri T, Taslimi Y, Abdossamadi Z, Heidari-Kharaji M, Gholami E, Sedaghat B, Niiyati M, Rafati S. Arginase activity in pathogenic and non-pathogenic species of *Leishmania* parasites. *PLoS Negl Trop Dis*. 2017;11(7):e0005774.
28. Hanedan B, Kirbas A, Kandemir FM, Ozkaraca M, Kilic K, Benzer F. Arginase activity and total oxidant/antioxidant capacity in cows with lung cystic echinococcosis. *Med Weter*. 2015;71(3):167–70.
29. Naseri M, Akbarzadeh A, Spotin A, Akbari NAR, Mahami-Oskouei M, Ahmadpour E. Scolicidal and apoptotic activities of albendazole sulfoxide and albendazole sulfoxide-loaded PLGA-PEG as a novel nanopolymeric particle against *Echinococcus granulosus* protoscoleces. *Parasitol Res*. 2016;115(12):4595–603.
30. De S, Pan D, Bera A, Sreevatsava V, Bandyopadhyay S, Chaudhuri D, Kumar S, Rana T, Das S, Das S. In vitro assessment of praziquantel and a novel nanomaterial against protoscoleces of *Echinococcus granulosus*. *J Helminthol*. 2012;86(1):26–9.
31. Hu H, Kang J, Chen R, Mamuti W, Wu G, Yuan W. Drug-induced apoptosis of *Echinococcus granulosus* protoscoleces. *Parasitol Res*. 2011;109(2):453–9.
32. Métayer LE, Brown RD, Carlebur S, Burke GA, Brown GC. Mechanisms of cell death induced by arginase and asparaginase in precursor B-cell lymphoblasts. *Apoptosis*. 2019;24(1):145–56.
33. Rodriguez-Garcia A, Hevia D, Mayo JC, Gonzalez-Menendez P, Coppo L, Lu J, Holmgren A, Sainz RM. Thioredoxin 1 modulates apoptosis induced by bioactive compounds in prostate cancer cells. *Redox Biol*. 2017;12:634–47.
34. Castillo J, Palomo-Canales J, Garcia J, Lastres J, Bolas F, Torrado J. Preparation and characterization of albendazole β -cyclodextrin complexes. *Drug Dev Ind Pharm*. 1999;25(12):1241–8.
35. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol*. 1992;54(2):165–73.
36. Spotin A, Majdi MMA, Sankian M, Varasteh A. The study of apoptotic bifunctional effects in relationship between host and parasite in cystic echinococcosis: a new approach to suppression and survival of hydatid cyst. *Parasitol Res*. 2012;110(5):1979–84.
37. Fetterer R. The effect of albendazole and triclabendazole on colchicine binding in the liver fluke *Fasciola hepatica*. *J Vet Pharmacol Ther*. 1986;9(1):49–54.

38. Garcia A, Leonardi D, Salazar MO, Lamas MC. Modified β -cyclodextrin inclusion complex to improve the physicochemical properties of albendazole. Complete in vitro evaluation and characterization. PLoS ONE. 2014;9:e88234.
39. Castillo JA, Palomo-Canales J, Garcia JJ, Lastres JL, Bolas F, Torrado JJ. Preparation and characterization of albendazole β -cyclodextrin complexes. Drug Dev Ind Pharm. 1999;25(12):1241–8.
40. Garcia-Rodriguez J, Torrado J, Bolas F. Improving bioavailability and anthelmintic activity of albendazole by preparing albendazole-cyclodextrin complexes. Parasite. 2001;8:188–90.
41. Ceballos L, Alvarez L, Sanchez Bruni S, Elisondo C, Dopchiz M, Denegri G, Torrado J, Lanusse C. Development of a cyclodextrin-based flubendazole formulation to control secondary echinococcosis: pharmacokinetics, hydatid cyst morphology and efficacy in mice. J Vet Pharmacol Ther. 2006;29:85–6.
42. Casulli A, Gomez Morales MA, Gallinella B, Turchetto L, Pozio E. 2-Hydroxypropyl- β -cyclodextrin improves the effectiveness of albendazole against encapsulated larvae of *Trichinella spiralis* in a murine model. J Antimicrob Chemother. 2006;58(4):886–90.
43. García A, Leonardi D, Vasconi MD, Hinrichsen LI, Lamas MC. Characterization of albendazole-randomly methylated- β -cyclodextrin inclusion complex and in vivo evaluation of its antihelminthic activity in a murine model of Trichinellosis. PloS one. 2014;9(11):e113296.
44. Codina AV, García A, Leonardi D, Vasconi MD, Di Masso RJ, Lamas MC, Hinrichsen LI. Efficacy of albendazole: β -cyclodextrin citrate in the parenteral stage of *Trichinella spiralis* infection. Int J Biol Macromol. 2015;77:203–6.
45. Aminpour S, Rafiei A, Jelowdar A, Kouchak M. Evaluation of the Protoscolicidal Effects of Albendazole and Albendazole Loaded Solid Lipid Nanoparticles. Iran J Parasitol. 2019;14(1):127.
46. Pensel PE, Gamboa GU, Fabbri J, Ceballos L, Bruni SS, Alvarez LI, Allemandi D, Benoit JP, Palma SD, Elisondo MC. Cystic echinococcosis therapy: Albendazole-loaded lipid nanocapsules enhance the oral bioavailability and efficacy in experimentally infected mice. Acta Trop. 2015;152:185–94.
47. Gamboa GVU, Pensel PE, Elisondo MC, Bruni SFS, Benoit J-P, Palma SD, Allemandi DA. Albendazole-lipid nanocapsules: Optimization, characterization and chemoprophylactic efficacy in mice infected with *Echinococcus granulosus*. Exp Parasitol. 2019;198:79–86.
48. Kang J, Hu H, Yuan W, Wu G, Chen R, Baishanbieke W, Aisaiti K. Apoptosis induced in vitro by dexamethasone and ATP in the protoscolex of *Echinococcus granulosus*. Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi = Chinese. J Parasitol parasitic Dis. 2009;27(4):332–5.
49. Ahmadpour E, Godrati-Azar Z, Spotin A, Norouzi R, Hamishehkar H, Nami S, Heydarian P, Rajabi S, Mohammadi M, Perez-Cordon G. Nanostructured lipid carriers of ivermectin as a novel drug delivery system in hydatidosis. Parasit Vectors. 2019;12(1):1–9.
50. Paredes R, Jimenez V, Cabrera G, Iragüen D, Galanti N. Apoptosis as a possible mechanism of infertility in *Echinococcus granulosus* hydatid cysts. J Cell Biochem. 2007;100(5):1200–9.

51. Martínez-Espinosa R, Argüello-García R, Saavedra E, Ortega-Pierres G. Albendazole induces oxidative stress and DNA damage in the parasitic protozoan *Giardia duodenalis*. Front Microbiol. 2015;6:800.
52. Bártíková H, Vokřál I, Skálová L, Lamka J, Szotáková B. In vitro oxidative metabolism of xenobiotics in the lancet fluke (*Dicrocoelium dendriticum*) and the effects of albendazole and albendazole sulphoxide ex vivo. Xenobiotica. 2010;40(9):593–601.
53. Rahman I, Biswas SK, Kode A. Oxidant and antioxidant balance in the airways and airway diseases. Eur J Pharmacol. 2006;533(1–3):222–39.

Figures

Figure 1

(A) Fertile hydatid cysts (containing PSCs) obtained from the liver of infected sheep slaughtered in Northwest Iran. (B) Light microscopy of *E. granulosus* PSCs after staining with 0.1% eosin. Dead PSCs absorbed eosin and colored red, but alive protoscolex remained colorless (C) Disrupted protoscolex due to apoptotic effect of ABZ- β -CD.

Figure 2

Fourier transform infrared spectra of **(A)** albendazole (ABZ), **(B)** β-CD and **(C)** ABZ-loaded β-CD.

Figure 3

(A) Zeta potential determination of ABZ- β -CD and (B) Scanning electron microscopy of ABZ-loaded β -CD.

Figure 4

(A) Scolicidal effects of ABZ and **(B)** ABZ- $\beta\text{-CD}$ against PSC of *Echinococcus granulosus sensu stricto* at the different concentrations (100, 200, 400 and 800 $\mu\text{g/mL}$) for 9 sequential days. ABZ, ABZ loaded $\beta\text{-CD}$ and $\beta\text{-CD}$ were dissolved in a final concentration of 0.1 % DMSO. PSC: Protoscolex.

Figure 5

Observation on PSC by SEM **(A)** treated with β -CD (control) during the treatment period, **(B)** PSC treated with 400 μ g/mL ABZ- β -CD indicated a disrupted characteristic due to apoptosis, **(C)** PSC treated with DMSO (0.1%), **(D)** PSC treated with 400 μ g/mL ABZ. H: Hook; CC: Calcareous corpuscles; DS: Disrupted surface; PSC: Protoscolex.

Figure 6

Assessment of Caspase-3 enzymatic activity in PSC treated with controls (PSC treated with 0.1% DMSO for ABZ group and PSC treated with β-CD in 0.1% DMSO for ABZ-β-CD group), ABZ and ABZ-loaded β-CD after 15 h of exposure time. Caspase-3 activity assays were repeated at least three times in triplicate. Asterisk (*); statistically indicating significant level ($P < 0.05$). PSC: Protoscolex.

Figure 7

(A) Fold-change values in mRNA expression levels of Arginase (*EgArg*) and **(B)** Thioredoxin peroxidase (*EgTPx*) of *E. granulosus* s.s. in PSC treated with controls (PSC treated with 0.1% DMSO for ABZ group and PSC treated with β-CD in 0.1% DMSO for ABZ-β-CD group), ABZ and ABZ-β-CD after 6 h of exposure time. All qPCR reactions were repeated in duplicate with employing elongation factor-1 alpha (*EF-1α*) as the housekeeping internal control. In this study, the mRNA expression of *EgTPx* in PSC treated with different concentrations of ABZ was not detected in repeated experiment. Asterisk (*); statistically indicating significant level ($P < 0.05$). PSC: Protoscolex.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Graphicalabstract.jpg](#)
- [Highlights.docx](#)