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Apoptotic Effects of Flubendazole-Loaded mPEG-PCL Nanoparticles on *Echinococcus granulosus* Protoscoleces

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ABSTRACT

Background and Aim: Despite using various drugs, none have proven fully effective in treating cystic echinococcosis. This study aimed to evaluate the apoptotic effects of flubendazole-loaded mPEG-PCL nanoparticles on *Echinococcus (E.) granulosus* protoscoleces.

Materials and Methods: Hydatid cysts were collected from the livers of infected sheep at the Zanjan slaughterhouse, and protoscoleces were aspirated from the fertile cysts. The viability of the collected parasites was determined using methylene blue exclusion test. Subsequently, the protoscoleces were treated for 48 hr with flubendazole-loaded mPEG-PCL nanoparticles at final concentrations of 5 and 10 µg/mL, as well as with free flubendazole at the same concentrations. To assess apoptosis in protoscoleces, the TUNEL assay and DAPI staining were employed. Nuclear morphological changes were then examined under fluorescence microscope. The Kruskal–Wallis test in R software was used to compare the apoptotic rates among different groups.

Results: The highest apoptotic rate was observed in protoscoleces exposed to flubendazole-loaded nanoparticles at 10 µg/mL concentration after 48 hr of incubation, reaching 63 ± 6.55%. In contrast, free flubendazole at the same concentration induced apoptosis in 42.66 ± 5.68% of protoscoleces.

Conclusion: Flubendazole-loaded mPEG-PCL nanoparticles exhibited greater apoptotic effects on *E. granulosus* protoscoleces compared to free flubendazole.

Keywords: Apoptosis, Cystic echinococcosis, *Echinococcus granulosus*, Flubendazole, Nanoparticles

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1. Introduction

Cystic echinococcosis (CE) is one of the most important zoonotic diseases worldwide. In this disease, the larval stage of *Echinococcus (E.) granulosus* leads to the formation of fluid-filled cysts, known as hydatid cysts, which primarily develop in the liver and lungs. The slow-growing, tumor-like expansion of hydatid cysts can cause severe functional impairment of the affected organs and may even result in patient death. The adult form of this parasite resides in the small intestine of canids (definitive hosts). Dogs infected with this worm excrete the eggs. If herbivores consume forage contaminated with the parasite's eggs or humans accidentally consume vegetables or

water contaminated with the eggs, the embryo initially emerges from the egg in the intestine. It travels through the bloodstream to various organs, where it develops into a hydatid cyst **(1, 2)**.

Currently, benzimidazole compounds, such as mebendazole and particularly albendazole, are used for the pharmacological treatment of CE. However, the efficacy of these drugs, especially under *in vivo* conditions, is limited **(3-5)**. Therefore, optimizing the formulation of existing anti-echinococcosis drugs and evaluating other benzimidazole derivatives appears essential for enhancing therapeutic outcomes in this disease.

Apoptosis is a programmed cell death process characterized by morphological changes, including chromatin condensation, nuclear fragmentation, and cell shrinkage. The most critical event in apoptosis is the cleavage of nuclear DNA into smaller fragments **(6, 7)**. Activating a process in protoscoleces that leads to their programmed death could significantly reduce the recurrence of cysts after treatment.

Flubendazole (FLBZ) ([5-(4-fluorobenzoyl)-1H-benzimidazole-2-yl]-carbamic acid methyl ester), a synthetic anthelmintic drug belonging to the benzimidazole carbamate group, is used for treating nematode infections in both humans and animals **(8)**. These compounds disrupt the parasite's energy metabolism by reducing the glucose uptake, ultimately leading to parasite death **(9, 10)**. Recent studies have reported that flubendazole induces cell death in solid tumour cell lines and breast cancer cells **(11, 12)**. Additionally, flubendazole exerts significant effects on protoscoleces **(13)** and has shown prophylactic effects in preventing secondary cyst formation in mice **(14)**. Elissondo et al **(15)** demonstrated that FLBZ at concentrations of 1, 5, and 10 µg/mL could eliminate *E. granulosus* protoscoleces *in vitro*. The highest protoscolicidal effect was observed at 10 µg/mL, resulting in 100% protoscoleces death after 30 days **(15)**. Pérez-Serrano et al **(16)** reported 50% mortality rate of protoscoleces after 24 days of incubation with ABZ and ABZ sulfoxide. However, the low solubility of FLBZ and its inadequate absorption in the human body limit its clinical efficacy **(17, 18)**. The use of nanoparticles has been proposed as an effective strategy to enhance the therapeutic efficacy of this drug. Moreover, nanoparticles can increase apoptotic effects in target cells by altering the structural and physical properties of the drug. Studies have shown that various metallic or polymeric nanoparticles, such as zinc and gold nanoparticles (Au-NCs) can induce programmed cell death in *E. granulosus* protoscoleces by activating apoptotic pathways *in vitro* **(19-22)**.

Given the critical role of apoptosis in programmed cell death and its potential advantage over the toxic effects of necrosis, focusing on the induction of cellular apoptosis is essential in antiparasitic drug studies. In our previous study, a novel flubendazole nanoparticle formulation was synthesized using biodegradable mPEG-PCL nanoparticles to evaluate its therapeutic effects on *E. granulosus* **(23)**. In the present study, the effects of flubendazole-loaded nanoparticles on the induction of apoptosis in *E. granulosus* protoscoleces were investigated *in vitro*.

2. Materials and Methods

2.1 Preparation and Characterization of Flubendazole-Loaded mPEG–PCL Nanoparticles

Flubendazole-loaded nanoparticles were prepared by a single step nano-precipitation method as described in our previous study **(23)**. Briefly, mPEG–PCL co-polymer and flubendazole (Santa Cruz Biotechnology, USA) were dissolved in pure acetone. The organic phase was then added drop wise into distilled water under sonication. The resulting suspension was stirred magnetically at 25°C and 180 rpm until the remaining acetone was completely evaporated. The obtained nanoparticle suspension was washed and re-suspended in distilled water prior to use. As previously reported **(23)**, the physicochemical characteristics of the prepared flubendazole-loaded nanoparticles were as follow: hydrodynamic diameter (z average size) ≈ 101.14 ± 5.14 nm, ζ potential ≈ –19.13 ± 2.56 mV, polydispersity index (PDI) ≈ 0.138 ± 0.04, and encapsulation efficiency (EE%) = 89.16 ± 2.93% (corresponding to a drug loading (DL) of ~ 3.08 ± 0.15%).

2.2 Collection of Protoscoleces

Livers infected with hydatid cysts were collected in May 2018 from slaughtered sheep at the Zanjan slaughterhouse, an area where *E. granulosus* sensu stricto is prevalent **(24)**. Protoscoleces were aspirated from the fertile hepatic cysts under sterile conditions and washed several times with phosphate-buffered saline (PBS, pH 7.4) containing penicillin and streptomycin using centrifugation. The viability of the collected parasites was assessed under a light microscope using the methylene blue (0.1%) exclusion test, as well as by observing the movement of rostellar hooks, flame cells, and the body of the protoscoleces **(25)**.

2.3 *In Vitro* Incubation and Apoptosis Assessment

To evaluate the apoptotic effects of free flubendazole and flubendazole-loaded nanoparticles on *E. granulosus* protoscoleces *in vitro*, a stock solution of free flubendazole (1 mg/mL) was prepared

by dissolving 1 mg of flubendazole in 1 mL of dimethyl sulfoxide (DMSO). Subsequently, 10 mL of RPMI 1640 medium containing 100 µg/mL streptomycin and 100 u/mL penicillin was added to each filtered culture flask. Approximately 2,000 active protoscoleces with over 95% viability were cultured in this medium and incubated at 37°C with 5% CO_2 for 48 hr. During this period, protoscoleces were treated with free flubendazole at final concentrations of 5 and 10 µg/mL, as well as with flubendazole-loaded nanoparticles at the same concentrations. The stated concentrations refer to the encapsulated flubendazole (i.e. active drug), not to the total nanoparticle mass. Also, two negative control groups were included: one containing mPEG-PCL nanoparticles without flubendazole, and the other, a culture medium containing 0.1 mg/mL DMSO.

The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) and DAPI (4',6-diamidino-2-phenylindole) methods were used to assess apoptosis in protoscoleces. After 24 and 48 hr of incubation, protoscoleces from both control and treatment groups were collected and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde at 4°C for 24 hr. Apoptosis detection was then performed according to the *In Situ* Cell Death Detection Kit (Fluorescein, Roche, Germany, and nuclei were stained with DAPI) **(26)**. Briefly, the samples were washed with PBS and incubated for 20 min at room temperature in 0.4% Triton X-100. After washing with PBS, samples were incubated with 0.1% proteinase K at 37°C for 20 min and then washed again with PBS. The enzyme solution and labelling solution provided in the TUNEL kit were added to the samples, which were incubated in the dark for 1 hr. Following PBS washing, protoscoleces were covered with DAPI at a final concentration of 1 µg/mL in PBS for nuclear staining and incubated in the dark at room temperature for 15 min, wrapped in foil. After removing the excess solution, a drop was placed on a glass slide and examined under fluorescence microscope for colorimetric changes. Apoptotic nuclei were observed as bright spots with high fluorescence intensity using TUNEL and DAPI staining. All experimental conditions were performed in three independent biological replicates, and for each replicate, at least 200 protoscoleces were counted manually to assess the apoptosis effects. Apoptotic rate was expressed as the percentage of TUNEL-positive protoscoleces relative to the total counted protoscoleces.

2.4 Statistical Analysis

Results are presented as mean ± standard deviation. Comparisons between groups were performed using R software (version 3.4.3). The Kruskal–Wallis test was used to evaluate differences in apoptosis levels among groups, and a P-value less than 0.05 was considered statistically significant.

3. Results

Apoptotic effect of Flubendazole-Loaded Nanoparticles on *E. granulosus* Protoscoleces

Protoscoleces collected from fertile hepatic cysts of infected sheep livers exhibited over 95% viability (Figure 1). Apoptosis in protoscoleces was evaluated by TUNEL assay. This method clearly revealed the presence of fragmented nuclear DNA in protoscoleces exposed to the drugs. Apoptotic nuclei appeared as bright spots with high fluorescence intensity following TUNEL and DAPI staining (Figures 2 and 3), whereas such bright spots were absent in healthy protoscoleces (Figures 4 and 5). Apoptosis rates of protoscoleces during the incubation period at different concentrations of flubendazole-loaded nanoparticles and free flubendazole are presented in Table 1. In contrast to flubendazole-loaded nanoparticles, no significant difference in apoptosis rate was observed in any of the free flubendazole groups compared to the control group after 24 hr of incubation. However, after 48 hr, significant differences were observed between control group and flubendazole-loaded nanoparticle groups (both at 5 and 10 µg/mL) as well as free flubendazole group (only at 10 µg/mL) (P<0.05) (Table 1). Following 48 hr exposure to flubendazole-loaded nanoparticles at 5 µg/mL, 39.66 ± 4.72% of protoscoleces underwent apoptosis (P<0.05), whereas in the same concentration of free flubendazole, apoptosis was 15.66 ± 2.08%. Although the increase in apoptotic protoscoleces in the 10 µg/mL flubendazole-loaded nanoparticle group (63 ± 6.55%) was higher and more pronounced than that of free flubendazole (42.66 ± 5.68%), no statistically significant difference was observed between two groups (P>0.05) (Table 1). Nevertheless, the flubendazole-loaded nanoparticles induced apoptosis in approximately 1.5-fold more protoscoleces than free drug at the same concentration (10 µg/mL), indicating a notable numerical trend. These results indicated that flubendazole is capable of inducing apoptosis in the nuclei of protoscoleces.

Table 1. Percentage of apoptotic parasites in the different treatment groups after 24 and 48 hr of incubation.

Groups	Percentage of apoptotic parasites after 24 hr (Mean ± SD)	Percentage of apoptotic parasites after 48 hr (Mean ± SD)
DMSO (control)	6 ± 2	6.66 ± 2.51
mPEG-PCL nanoparticles (control)	5 ± 2.65	5.33 ± 1.52
Free FLBZ 10 µg/mL	17.33 ± 3.51	*42.66 ± 5.68
Free FLBZ 5 µg/mL	12.33 ± 3.1	15.66 ± 2.08
FLBZ-loaded nanoparticles 10 µg/mL	22 ± 2.65*	*63 ± 6.55
FLBZ-loaded nanoparticles 5 µg/mL	21 ± 7.21*	*39.66 ± 4.72

*** *P*<0.05: indicates a statistically significant difference between the treatment groups and the control group. Note: FLBZ = flubendazole; NPs = mPEG-PCL nanoparticles; SD = standard deviation.**

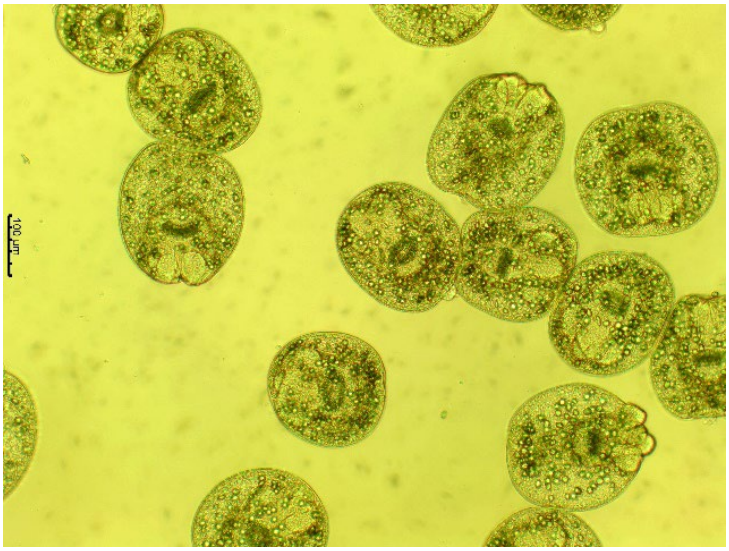


Figure 1. *E. granulosus* protoscoleces. Image shows *E. granulosus* protoscoleces collected from hydatid cysts of infected sheep that exhibited over 95% viability **(Prepared by Authors, 2025)**.

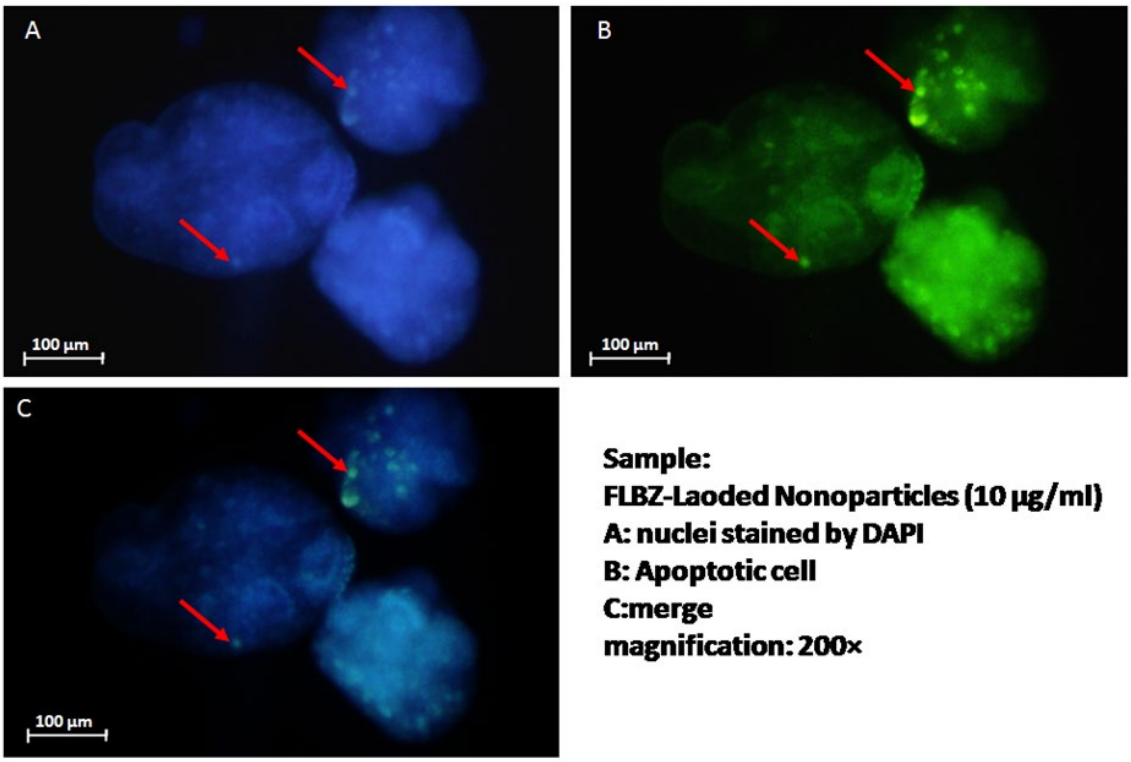


Figure 2. Apoptotic cells in treated protoscoleces. The images show DAPI in blue (A), TUNEL in green (B), and merged DAPI and TUNEL staining of nuclei (C) in treated protoscoleces. Apoptotic cells are observed as bright spots with high fluorescence intensity following TUNEL (bright green fluorescence) and DAPI (blue fluorescence) staining. Arrows point to representative apoptotic cells exhibiting characteristic of nuclear condensation and fragmentation **(Prepared by Authors, 2025)**.

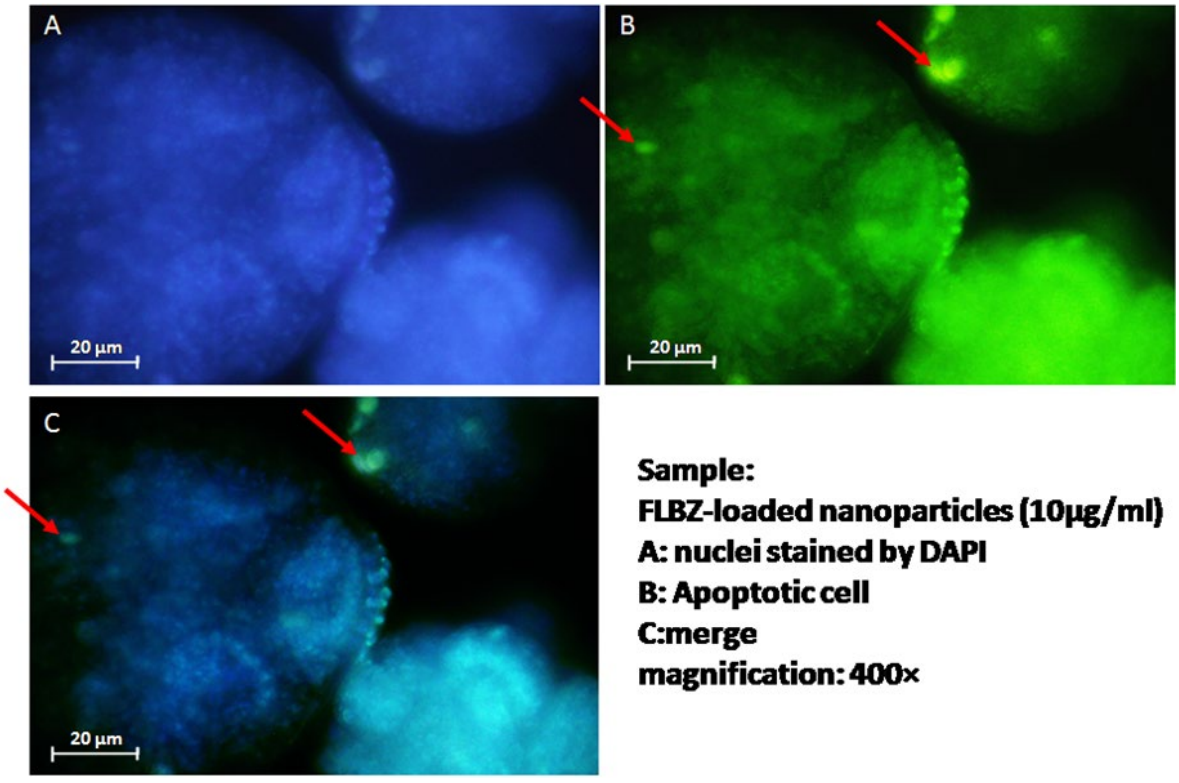


Figure 3. Higher magnification of apoptotic cells in treated protoscoleces. Apoptotic cells are visualized as bright spots with high fluorescence intensity following TUNEL and DAPI staining. Arrows point to representative apoptotic cells exhibiting characteristic of nuclear condensation and fragmentation **(Prepared by Authors, 2025)**.

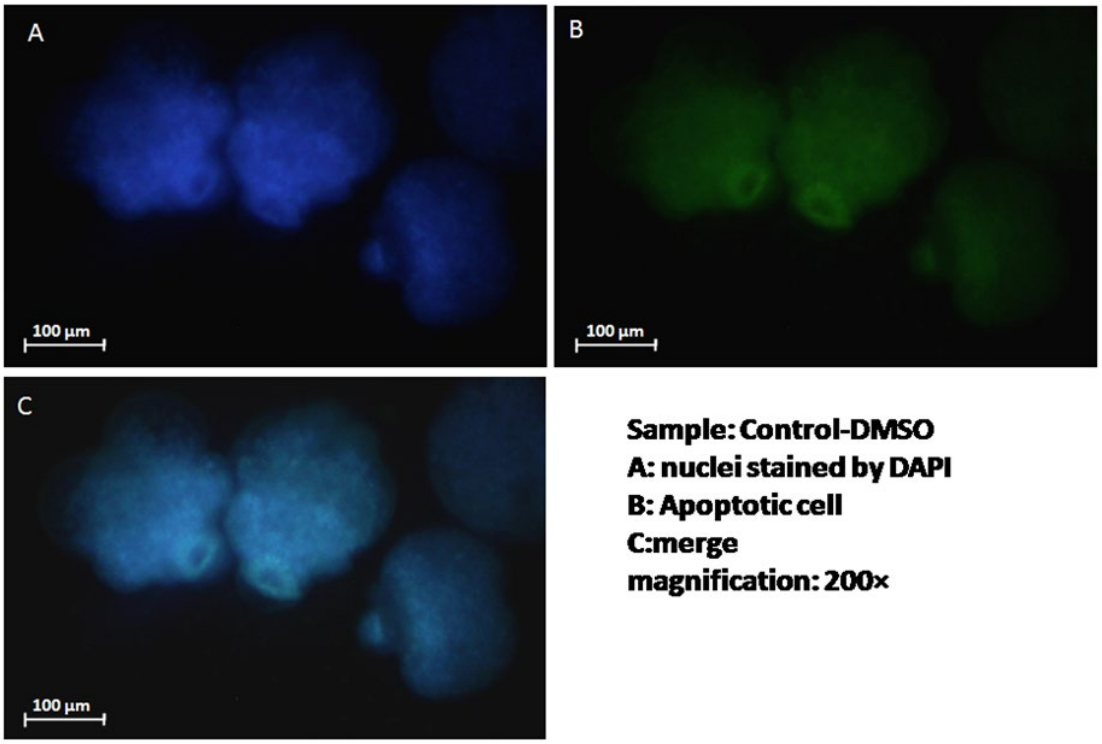


Figure 4. Untreated protoscoleces. The images show DAPI in blue (A), TUNEL in green (B), and merged DAPI and TUNEL staining of nuclei (C) in untreated (DMSO control group) protoscoleces. The nuclei stained with DAPI exhibit uniform blue fluorescence, and TUNEL staining shows no green bright spots (indicative of DNA fragmentation). In normal protoscoleces, apoptotic cells with bright fluorescence spots were not observed **(Prepared by Authors, 2025)**.

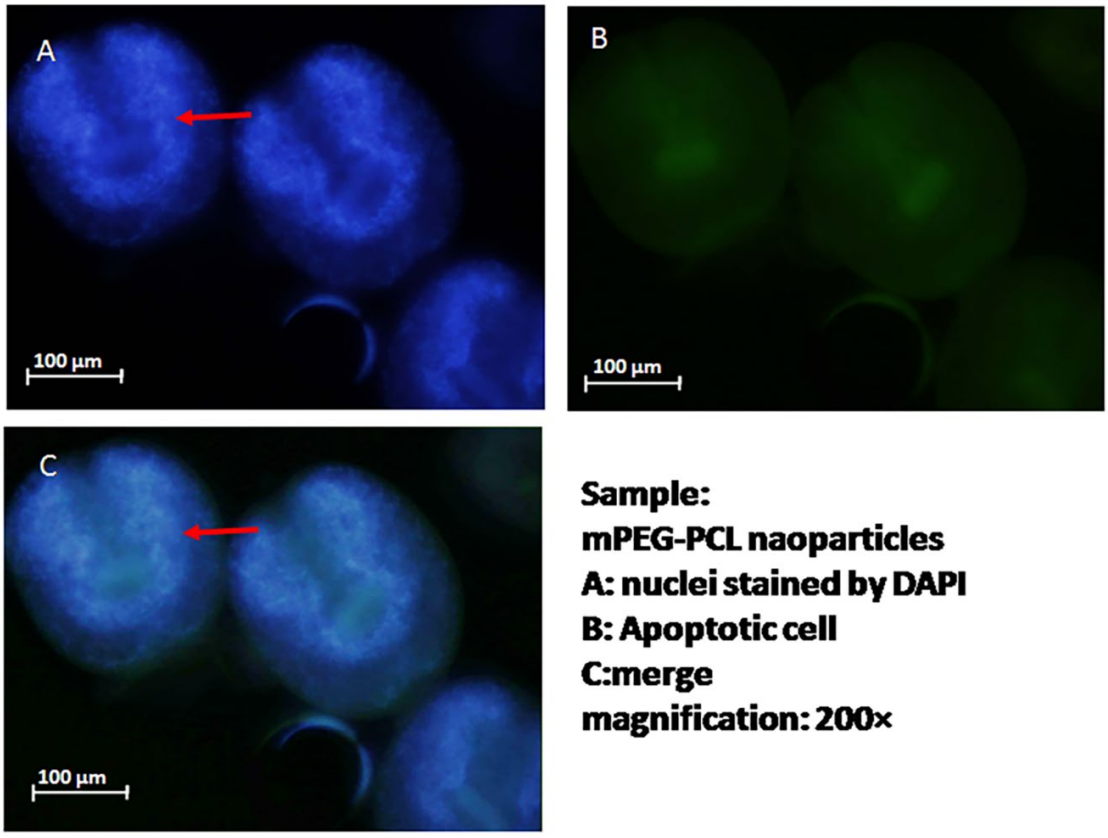


Figure 5. Nuclei of parasite cells with intact, non-apoptotic DNA in control protoscoleces. In untreated control protoscoleces, nuclei appear uniform and show no bright TUNEL-positive spots. Arrows indicate nuclei with intact DNA that show no signs of apoptosis **(Prepared by Authors, 2025)**.

4. Discussion

Cystic echinococcosis is recognized as a major public health problem in many countries, including Iran, particularly in rural and livestock-rearing areas. This disease can lead to severe complications and even death. Due to its apparently unlimited growth and proliferation, the hydatid cyst exhibits tumor-like characteristics, including the parasite's ability to modulate host immune responses and potentially disseminate, allowing it to progressively expand in various human organs **(27, 28)**. It has been demonstrated that flubendazole, commonly used for treating parasitic infections, plays a significant role in inhibiting cell growth in leukemia and intestinal cancer. Flubendazole affects the viability of leukemia and myeloma cell lines, and represents a potential therapeutic option for neuroblastoma, including treatment of drug-resistant cells **(29, 30)**.

In limited studies, the effect of flubendazole on *E. granulosus* protoscoleces has been investigated. In a study conducted by Elissondo et al **(15)**, a pronounced protoscolicidal effect of flubendazole was observed. After 30 days of exposure to 10 µg/mL flubendazole *in vitro*, protoscoleces mortality reached 100% **(15)**. When compared with ABZ, flubendazole demonstrated higher efficacy. Flubendazole at 10 nmol/mL caused 100% protoscoleces death on day 30, whereas approximately 70.9% of protoscoleces were killed 30 days post-incubation with ABZ **(13)**. However, low aqueous solubility and poor gastrointestinal absorption of flubendazole limit its therapeutic efficacy in humans **(31)**.

Recent studies have reported favourable outcomes using nanotechnology-based drug delivery systems for antiparasitic chemotherapy **(32-35)**. It has been shown that flubendazole-loaded nanoparticles (nanoformulation) are more effective than free flubendazole both *in vitro* and *in vivo* against protoscoleces and metacestodes of *E. granulosus*, leading to faster parasite killing **(23)**. Moreover, nanoparticles can enhance apoptotic effects in target cells by altering the physical and structural properties of the drug. The result of a recent study demonstrated that magnetic iron oxide nanoparticles coated with PO (FOMNPsP) significantly reduced the number, size, and weight of hydatid cysts. A strong protoscolicidal effect was observed at 400 µg/mL within 10 min, which was accompanied by upregulation of caspase-3 expression, indicating the induction of apoptosis in the parasite **(36)**. Nanoparticle-based drug delivery systems, such as mPEG-PCL or lipid/metal nanostructures, continually demonstrate higher efficacy compared to free drugs due to improved solubility, increased uptake by the parasite, and sustained release of the active compound. Encapsulation of flubendazole in mPEG-PCL nanoparticles can take advantage of their nanoscale size and high surface-to-volume ratio, which can enhance close contact or adsorption onto the parasite surface and thereby enhance local drug delivery, ensuring stable exposure of protoscoleces to cytotoxic concentrations over the incubation period. In this study, the apoptotic effects of flubendazole-loaded nanoparticles on *E. granulosus* protoscoleces were confirmed for the first time using the TUNEL assay. This method is based on the ability of the terminal deoxynucleotidyl transferase (TdT) enzyme to label free 3'-hydroxyl ends of DNA, which are generated due to single- and double-strand cleavage of chromatin during apoptosis or programmed cell death. These labeled nucleotides can be detected using fluorescent dyes, allowing the visualization of nuclei with fragmented DNA **(37)**.

Protoscoleces exposed to flubendazole-loaded nanoparticles exhibited greater apoptotic effects compared to free flubendazole, and the rate of apoptosis increased with both longer exposure time and higher drug concentrations. The maximum apoptotic effect was observed with 10 µg/mL FLBZ-loaded nanoparticles (P<0.05), where the percentage of apoptotic protoscoleces reached 22 ± 2.65% at 24 hr and increased to 63 ± 6.55% at 48 hr (P<0.05). Although 10 µg/mL free flubendazole also induced a significant apoptotic effect on protoscoleces (P<0.05), this effect was approximately 1.5-fold lower than that of flubendazole-loaded nanoparticles. Specifically, 48 hr post-incubation with 10 µg/mL free flubendazole, the apoptosis rate reached 42.66 ± 5.68%.

A few studies have investigated the effects of drugs on induction of apoptosis in *E. granulosus* protoscoleces. Moghadaszadeh et al **(38)** evaluated the protoscolicidal and apoptotic effects of various concentrations of liposomal nanoparticles containing 5-hydroxy-1,4-naphthoquinone (Juglone) on *E. granulosus* protoscoleces *in vitro*. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to detect DNA damage and induction of apoptosis. The mRNA expression of caspase-3, which is essential for DNA fragmentation, was higher in protoscoleces treated with Juglone-loaded nanoliposomes for 15 hr compared to control groups **(38)**. Following exposure of protoscoleces to praziquantel-containing nanomaterials, activation of caspase-3 was demonstrated using colorimetric assays, and regular DNA fragmentation into small pieces further confirmed apoptosis. In comparison, when praziquantel was used alone, protoscoleces exhibited more necrotic features than apoptotic ones **(39)**.

In the present study, in addition to flubendazole-loaded nanoparticles at 5 and 10 µg/mL, free

flubendazole also induced significant apoptosis in protoscoleces after 48 hr compared to the control group. Under these experimental conditions, flubendazole appeared to induce higher apoptotic activity than praziquantel, although these observations are limited to *in vitro* model and cannot be directly extrapolated to therapeutic effects.

In another study, the chloroform extract of *Astragalus onobrychis* was investigated for its apoptotic and DNA-damaging effects on protoscoleces using protease colorimetric assays and Real-Time PCR analysis. Due to apoptosis induction by this extract, significant activation of caspase-3 was observed in protoscoleces, accompanied by DNA damage and increased expression of the EgATM and EgP53 genes **(40)**. The use of a β-cyclodextrin carrier for albendazole leads to enhanced effect of the drug; compared to the free form, it markedly increases caspase-3 activity in *E. granulosus* protoscoleces, resulting in greater induction of apoptosis **(21)**. The use of copper nanoparticles (CuNPs), either alone or in combination with albendazole, has been shown to exhibit significant protoscolicidal effects by activating apoptotic pathways and increasing caspase activity in protoscoleces **(22)**. In a study conducted by Hosseini et al **(41)** antigens of laminated layers were used to induce apoptosis and expression of key genes in the cell proliferation pathway in A549 cell line. A549 cells were incubated in DMEM culture medium with different concentrations of hydatid cyst laminated layer (LL) antigens for 24 hr. Flow cytometry was used to measure apoptosis. Real-Time PCR was used to examine the expression levels of SOX-9, β-catenin, CD133, and CD44 genes. According to the results obtained from flow cytometry, treatment with LL antigens increased the rate of apoptosis in cells. The number of apoptotic cells increased significantly with increasing antigen concentration. The decrease in expression of the studied genes (except SOX-9) was also statistically significant **(41)**. Wang et al **(42)** investigated the role of Fis1 and PDCD6 genes in apoptosis of *E. granulosus* protoscoleces. RNA interference (RNAi) was used to reduce the expression of the studied genes in protoscoleces, and the apoptosis rate was examined using the TUNEL method and apoptotic cell counting in different groups. Due to decrease in the expression of Fis1 and PDCD6 genes, an increase was observed in the apoptosis rate in protoscoleces. The apoptosis rate in the Fis1-IG and PDCD6-IG groups was 53.85% and 65.07%, respectively, while the apoptosis rate in the negative control group was only 13.95% **(42)**.

In this study, the TUNEL assay was used to confirm apoptosis induced in protoscoleces. Only a few limited studies have employed the TUNEL method to demonstrate apoptosis in protoscoleces. Kang et al **(43)** incubated protoscoleces for 8 hr with dexamethasone (5 mmol/L) + ATP (1.6 mmol/L). TUNEL staining indicated the presence of apoptotic cells, and caspase-3 activity increased in protoscoleces 12-fold compared to the control group **(43)**. Similarly, for apoptosis assessment using TUNEL and caspase-3 expression via immunohistochemistry, protoscoleces were exposed to hydrogen peroxide (H_2O_2, 1 mM) and dexamethasone (5 mM) for 8 hr, which induced apoptosis in protoscoleces **(44)**.

While this study tested only two concentrations of FLBZ-loaded mPEG-PCL nanoparticles, which limits dose–response analysis and prevents IC_{50} estimation, the results clearly demonstrate a significant apoptotic effect at the tested doses. Future studies should evaluate a broader range of concentrations to better define the dose–response curve and optimize therapeutic dosing.

5. Conclusion

In the present study, flubendazole-loaded nanoparticles at both 5 and 10 µg/mL induced significant apoptosis at 24 and 48 hr, whereas free flubendazole only exhibited a significant apoptotic effect at 10 µg/mL after 48 hr compared with control group. Therefore, the results indicate that flubendazole is capable of inducing apoptosis in protoscoleces nuclei, and this effect is more pronounced when delivered via flubendazole-loaded nanoparticles compared to free flubendazole.

6. Declarations

6.1 Acknowledgment

The authors are grateful to the faculty members of the Anatomy Department, Zanjan Medical School, for their valuable guidance.

6.2 Ethical Considerations

This study was approved by the Ethics Committee of Zanjan University of Medical Sciences (approval code: ZUMS.REC.1394.133).

6.3 Authors' Contributions

Ali Haniloo, Kobra Rostamizadeh, and Mehdi Farhadi conceived the idea, designed the study, and performed the experiments. All authors analyzed the data and reviewed the results. Mehdi Farhadi and Saeid Amanloo drafted the final version of the manuscript. All authors have read and approved the final version.

6.4 Conflict of Interests

The authors have no conflicts of interest.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this research.

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