

Original Article



Enhanced Protoscolicidal Activity of Pulsed Electric Fields Combined With Albendazole: An in Vitro Study

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Abstract

Introduction: Cystic echinococcosis (CE), caused by the metacestode stage of *Echinococcus granulosus*, remains a significant zoonotic disease with serious health and economic burden worldwide, especially in endemic regions. Despite the availability of surgical and pharmacological treatments, complications such as cyst content leakage and recurrence are prevalent, necessitating novel and more effective therapeutic approaches. Accordingly, this study aimed to evaluate the synergistic protoscolicidal effects of pulsed electric fields (PEF) and albendazole (ALB).

Methods: Protoscoleces were aseptically extracted from hydatid cysts in sheep livers and exposed to PEF at intensities of 70, 150, and 1300 V/cm for 15–60 minutes. Experimental groups included (1) PEF alone, (2) PEF combined with ALB, and (3) ALB alone. Moreover, viability of protoscoleces was assessed using the standard eosin exclusion assay.

Results: PEF alone increased mortality in a time-dependent and intensity-dependent manner, reaching 100% at 60 minutes. In addition, PEF+ALB (100 µg/mL) and ALB alone (100 µg/mL) achieved 100% mortality at 30–45 and 60 minutes, respectively. Nonetheless, PEF+ALB significantly outperformed PEF alone at early time points: 70 V/cm/15 minutes (72.03% vs. 87.6%, $P<0.01$), 1300 V/cm/15 minutes (86.79% vs. 95%, $P<0.05$), and 1300 V/cm/30 minutes (94.3% vs. 100%, $P<0.01$).

Conclusion: While these findings provide preliminary in vitro proof-of-concept for a synergistic effect, clinical application remains premature. Thus, further investigations must prioritize experiments with clinically relevant ALB concentrations, ex vivo studies on intact cysts, and in vivo validation. The clinical applicability of PEF in CE management requires extensive further research.

Keywords: Pulsed Electric Fields, Cystic echinococcosis, Protoscoleces, Albendazole



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Introduction

Cystic echinococcosis (CE), also known as hydatidosis, is a major zoonotic infection that is caused by the larval stage of *Echinococcus granulosus*, with a high prevalence rate particularly in regions with extensive livestock farming (1, 2). Humans acquire the infection by ingesting eggs excreted in the feces of canids, primarily through the fecal-oral route. It should be noted that the liver is the most commonly affected organ (3, 4). The global economic burden of CE

is estimated at approximately USD 3 billion annually, accounting for both medical expenses and substantial losses in the livestock sector (5, 6). In Iran, an estimated USD 232.25 million is annually spent on CE, with a significant portion attributed to livestock-related losses (7). Surgery remains the basis of CE treatment; however, it carries a couple of risks, such as cyst content leakage and disease recurrence. Moreover, the success of surgical interventions depends on the cyst's stage, size, location, and associated



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complications. Likewise, alternative therapies, including chemotherapy with benzimidazoles, such as mebendazole and albendazole (ALB), as well as the puncture, aspiration, injection, and re-aspiration technique, have limitations. They encompass biliary tract complications and challenges in managing multiple cysts concurrently (8, 9). Despite the availability of alternative treatments, surgery remains the most effective approach, provided that potent protoscolicidal agents are used to prevent recurrence. The intraoperative leakage of viable protoscoleces is a major factor that contributes to its recurrence (10). Although recurrence occurs in approximately 6.5% of cases, its impact can be serious, frequently requiring prolonged recovery and follow-up care (11, 12). The injection of protoscolicidal agents into hepatic CE is a critical step in surgery aimed at minimizing the risk of viable protoscoleces leakage. Commonly used protoscolicidal agents (e.g., hypertonic saline, mannitol, ALB, silver nitrate, hydrogen peroxide, ethyl alcohol, cetrimide, and some plant extracts) are often associated with adverse effects, limiting their clinical applicability (13-15). This situation highlights the urgent need for novel protoscolicidal agents that are fast-acting, effective at low doses, stable in cystic fluid, and resistant to dilution. Additionally, these agents should be capable of rapidly eliminating protoscoleces within the cyst. As a result, the management of CE remains both costly and complex, frequently necessitating extensive surgical interventions or long-term pharmacological treatments (16). The development of innovative and effective therapies is essential due to increasing resistance to traditional treatments, the complications related to these treatments, high rates of disease recurrence, and the limited efficacy of some current methods (17). Pulsed electric fields (PEF) represent an emerging technology capable of altering intracellular calcium concentrations and modulating critical cellular processes, such as apoptosis and cell proliferation. Evidence suggests that the increase in intracellular Ca^{2+} concentration may result from the formation of membrane pores; however, the exact location and mechanisms involved in this process remain under investigation and debate (18). During electroporation, cells exhibit increased uptake of drugs, genetic materials, proteins, and other biomolecules. According to research, adjusting key parameters, such as pulse number, peak voltage, and pulse frequency, can significantly affect membrane permeability. Consequently, PEF has been effectively applied in biomedical fields, including targeted drug delivery, gene therapy, and cancer treatment (19-22). In the context of electroporation applications in parasitology, a number of researchers have demonstrated that PEFs are effective in inactivating *Anisakis* spp. larvae in fish and *Trichinella* spp. larvae in pigs (23-25). Furthermore, some studies have investigated the effect of PEF on CE and its protoscoleces, demonstrating the potential effectiveness of PEF in parasite inactivation (26-28). Therefore, this study seeks to investigate the in vitro effects of combined PEF and ALB to evaluate their

protoscolicidal efficacy.

Materials and Methods

Collection of Protoscoleces

Protoscoleces were collected from the livers of sheep naturally infected with *Echinococcus granulosus sensu stricto* (genotype G1), which is the predominant strain in Iranian sheep. The infected livers were obtained from slaughtered animals at the Rasht abattoir in northern Iran. After sedimentation, the supernatant was carefully removed from the CE fluid, which had previously been aseptically transferred into 50 mL Falcon tubes. Subsequently, the sedimented protoscoleces were washed three times with normal saline. In addition, their viability was assessed by staining them with a 0.1% eosin solution and examining them under a light microscope (29). A stock suspension of viable protoscoleces was then prepared at a density of 100,000 protoscoleces/mL (10,000 protoscoleces in 100 μL). For PEF treatment, 50 protoscoleces were taken from this stock and suspended in 500 μL of normal saline.

Evaluation of Protoscolicidal Effects

This study utilized PEF with a square wave shape generated by a Pars Tarava device. Parameters were 70 V/cm and 150 V/cm (100 μs pulse width, 4000 pulses, 5 kHz each), and 1300 V/cm (100 μs pulse width, 8 pulses, 1 Hz). Furthermore, 50 protoscoleces were placed in each well to evaluate the protoscolicidal efficacy of PEF, and all experiments were performed in triplicate. The experimental groups included (1) protoscoleces exposed to PEF, (2) protoscoleces exposed to PEF immediately followed by the addition of ALB directly to the same cuvette (final concentration: 100 $\mu\text{g/mL}$), (3) positive control: protoscoleces exposed to ALB alone (100 $\mu\text{g/mL}$), and (4) negative control: untreated protoscoleces.

Staining and Morphological Analysis

A 0.1% eosin solution was used to assess the viability of the protoscoleces in the experimental groups. Non-viable protoscoleces absorbed the eosin dye and appeared red. In contrast, viable protoscoleces remained unstained and showed muscular movement and flame cell activity. Finally, the viability and mortality rates of the protoscoleces were recorded at 15 minutes, 30 minutes, 45 minutes, and 60 minutes following exposure to PEF and ALB in the experimental groups.

Statistical Analysis

The obtained data were statistically analyzed using GraphPad Prism, version 9.0. For each treatment group (PEF alone and PEF+ALB separately), two-way analysis of variance (ANOVA) was used to evaluate the effects of pulse intensity (70 V/cm, 150 V/cm, and 1300 V/cm) and exposure time (15 minutes, 30 minutes, 45 minutes, and 60 minutes), as well as their interaction. Additionally, Tukey's HSD post-hoc test was applied for multiple pairwise comparisons. All data are presented as means $\pm$ standard

deviations (SD), and a P -value < 0.05 was considered statistically significant.

Results

Microscopic analysis of protoscolexes exposed to different PEF intensities revealed a progressive increase in mortality rates over time (Figure 1). At 70 V/cm, mortality rates reached 72.03%, 83.07%, 90.76%, and 100% at 15 minutes, 30 minutes, 45 minutes, and 60 minutes, respectively. At 150 V/cm, these rates were 79.62%, 88.8%, 96.29%, and 100% at the corresponding time points. At the highest intensity (1300 V/cm), mortality reached 86.79% at 15 minutes, 94.3% at 30 minutes, and 100% at both 45 minutes and 60 minutes. The findings demonstrated that increased pulse intensity reduces protoscolex viability more rapidly,

with higher intensities achieving complete mortality sooner (Table 1, Figure 2). In the combined treatment group (PEF followed by ALB at a final concentration of 100 $\mu\text{g/mL}$), mortality rates were markedly higher. Likewise, mortality reached 87.6%, 90.7%, and 100% at 15 minutes, 30 minutes, and 45–60 minutes, respectively, following 70 V/cm pulses. The corresponding rates were 89.8%, 93.2%, and 100% at 150 V/cm. Notably, at 1300 V/cm, mortality reached 95% within 15 minutes and 100% thereafter (Table 2, Figure 3). Based on the results, combining PEF with ALB could significantly enhance protoscolex mortality compared to PEF alone. In the group treated with ALB alone (100 $\mu\text{g/mL}$), complete mortality was achieved within 60 minutes, confirming the drug's efficacy. Two-way ANOVA performed for each treatment

Table 1. Mortality Rates of Protoscolexes Exposed to PEF at 70, 150, and 1300 Intensities During 15–60 Minutes

Exposure Time (Minute)	Mortality Rate (%) at 70 PEF Intensity	Mortality Rate (%) at 150 PEF Intensity	Mortality Rate (%) at 1300 PEF Intensity
15	72.03	79.62	86.79
30	83.07	88.8	94.3
45	90.76	96.29	100
60	100	100	100

Note. PEF: Pulsed electric field

Table 2. Mortality Rates of Protoscolexes Exposed to PEF at 70, 150, and 1300 Intensities Plus and ALB (100 $\mu\text{g/mL}$) During 15–60 Minutes

Exposure Time (Minute)	Mortality Rate (%) at 70 PEF Intensity	Mortality Rate (%) at 150 PEF Intensity	Mortality Rate (%) at 1300 PEF Intensity
15	87.6	89.8	95
30	90.7	93.2	100
45	100	100	100
60	100	100	100

Note. PEF: Pulsed electric field

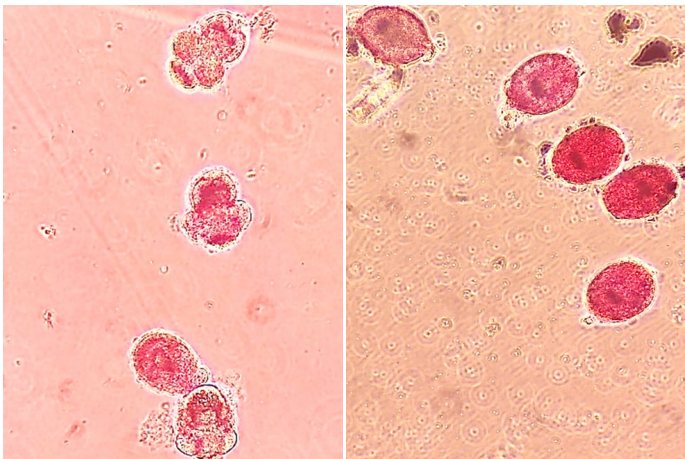


Figure 1. Microscopic Visualization of Dead Protoscolexes Stained With 0.1% Eosin Following PEF Exposure After 1 Hour (Left) and PEF Exposure Combined With ALB After 45 Minutes (Right)

Note. PEF: Pulsed electric field; ALB: Albendazole

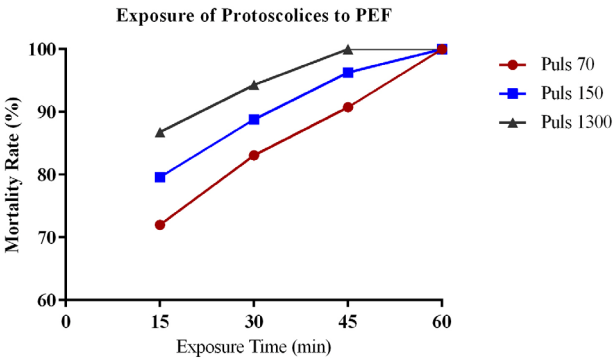


Figure 2. Mortality Rates of Protoscolexes Exposed to PEF at 70, 150, and 1300 Intensities During 15–60 Minutes

Note. PEF: Pulsed electric field; ALB: Albendazole

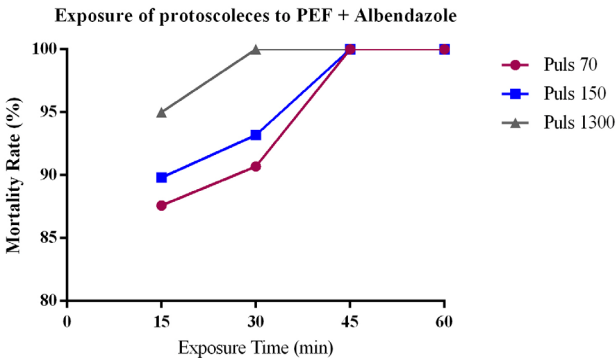


Figure 3. Mortality Rates of Protoscolexes Exposed to PEF at 70, 150, and 1300 Intensities Plus ALB (100 $\mu\text{g/mL}$) During 15–60 Minutes

Note. PEF: Pulsed electric field; ALB: Albendazole

group confirmed the significant effects of pulse intensity [PEF alone: $F(2, 24) = 34.67$, $P < 0.0001$; PEF+ALB: $F(2, 24) = 28.94$, $P < 0.0001$] and exposure time [PEF alone: $F(3, 24) = 42.35$, $P < 0.0001$; PEF+ALB: $F(3, 24) = 38.23$, $P < 0.0001$]. Similarly, remarkable interactions were observed between intensity and time [PEF alone: $F(6, 24) = 6.78$, $P < 0.001$; PEF+ALB: $F(6, 24) = 5.92$, $P < 0.01$]. Tukey's HSD post-hoc test revealed that mortality in the PEF+ALB group was significantly higher than in the PEF alone group at 15 minutes with 70 V/cm (72.03% vs. 87.6%, $P < 0.01$), at 15 minutes with 1300 V/cm (86.79% vs. 95%, $P < 0.05$), and at 30 minutes with 1300 V/cm (94.3% vs. 100%, $P < 0.01$). However, at 45 minutes and 60 minutes, no significant differences were found between the two groups at any intensity, as both reached 100% mortality. Overall, both longer exposure duration and higher pulse intensity substantially enhanced protoscolex mortality, underscoring the synergistic potential of combining physical and pharmacological treatments.

Discussion

CE is a zoonotic disease and is recognized as a major clinical issue in animals and humans worldwide, especially in regions where subsistence farming and animal husbandry form essential parts of the community (30, 31). Due to the low success rate of chemotherapy, surgery remains the preferred treatment for CE (9, 32–34). However, this treatment also poses certain challenges, and post-surgical medication is typically used to prevent complications (35). The pathological significance of CE has consistently driven researchers to explore effective methods for its control and treatment. To this end, different researchers have investigated the efficacy of various protoscolicidal agents, including hypertonic saline (36, 37), cetrimide (38, 39), chlorhexidine gluconate (40, 41), plant extracts (15, 42), and synthetic pharmaceuticals, such as ALB. In most studies, the viability of protoscoleces was evaluated using eosin staining; nonetheless, other methods (e.g., differential interference contrast/Nomarski microscopy) have also been utilized to improve assessment accuracy (43). Despite the potential of different protoscolicidal agents, the application of these compounds is constrained by various limitations, such as side effects from prolonged use, the emergence of drug resistance, ALB-associated toxicity, and ongoing debates regarding their overall effectiveness (44, 45). Therefore, the development of new protoscolicidal agents with no local or systemic side effects and higher efficacy is an urgent need for surgeons in order to ensure successful CE surgeries. PEF employs ultra-short electric pulses to interact with biologically active cells, affecting their membrane structure. The application of PEF causes charge imbalances on both sides of the cell membrane, leading to its deformation (43, 46–48). It should be noted that the effects of PEF on cells depend on pulse duration, electric field intensity, and pulse frequency, resulting in various intracellular biological responses (18). CE provides a closed system for studying the biological effects of PEF (26). Nonetheless, the application of PEF to various stages of CE

requires further extensive research. According to studies, PEF exerts its effects through disrupting the inner layers of the cyst, inducing an inflammatory zone around the cyst, promoting protoscolex death within the cyst, altering membrane permeability to facilitate drug penetration, detaching the germinal layer from the stratum corneum, and modifying the cyst's internal composition. Notably, PEF offers several advantages, including the preservation of the cyst's outer layer integrity, minimal damage to surrounding tissues, reduction of secondary infections, and the ability to be applied multiple times. Consequently, the use of PEF in the treatment of CE can contribute to the development of therapeutic strategies for this disease (20, 22, 26–28). Previous studies on nanosecond PEF have shown its scolical effect on human protoscoleces at 21–29 kV/cm and 100 pulses, causing membrane damage and enzyme leakage. In comparison, using conventional PEF (70–1300 V/cm), our study demonstrated that combining PEF with ALB achieves 100% mortality more rapidly (30–45 minutes) than PEF alone (45–60 minutes), confirming a synergistic effect even at lower field strengths (28). The exposure of protoscoleces to PEF at intensities of 70 V/cm, 150 V/cm, and 1300 V/cm revealed a clear dose-response relationship and a time-dependent increase in mortality. Mortality rates increased from 72.03% at 15 minutes to 100% at 60 minutes for 70 V/cm, from 79.62% at 15 minutes to 100% at 60 minutes for 150 V/cm, and from 86.79% at 15 minutes to 100% at 45 minutes for 1300 V/cm. Nevertheless, it is recommended that further studies perform safety evaluation for the 1300 V/cm parameter before intraoperative application, unlike 70 V/cm and 150 V/cm, which are within commonly reported safe ranges. These findings are consistent with those of prior research, emphasizing the ability of PEF to impair cellular membranes, induce irreversible electroporation, and increase cellular susceptibility to subsequent therapeutic interventions (27, 49, 50). In contrast, the second group (PEF+ALB) achieved mortality rates of 87.6% at 15 minutes to 100% at 45 minutes for 70 V/cm, 89.8% at 15 minutes to 100% at 45 minutes for 150 V/cm, and 95% at 15 minutes to 100% at 30 minutes for 1300 V/cm. Accordingly, the findings from the second group of this study underscore the potential of combining PEF with ALB as an effective strategy to enhance protoscolex mortality. The results also demonstrated a synergistic interaction between the two treatments, leading to significantly higher mortality rates compared to PEF treatment alone in the first group. Likewise, this combined approach proved particularly effective at higher pulse intensities, resulting in a shorter time to achieve complete protoscolex mortality. Furthermore, the increased efficacy observed in the combined treatment group suggests that PEF may function as a sensitizing agent by enhancing cell membrane permeability and facilitating the uptake of ALB (20–22). Our findings indicated that the second group performed significantly better than the first, representing a dose-dependent and time-dependent enhancement in efficacy due to the synergistic effect of the combined therapy.

The synergistic interaction detected in this study can be attributed to PEF's ability to induce structural and functional disruptions in the membrane of protoscoleces. Such disruptions likely enhance the penetration and intracellular action of ALB within the protoscoleces, thereby enhancing its parasitocidal efficacy. Furthermore, the rapid achievement of complete mortality in the combined treatment group implies that integrating physical and chemical interventions can overcome the potential limitations of each approach when used independently. From a practical perspective, the combination of PEF and ALB offers several advantages, including allowing lower drug concentrations or shorter treatment durations, potentially reducing the development of drug resistance and the occurrence of side effects. In addition, the rapid and effective mortality rates observed in this study suggest that this approach can be particularly beneficial in clinical settings where timely and effective treatment is critical. Hence, future studies should focus on further elucidating the mechanisms underlying the observed synergy between PEF and ALB. Moreover, investigating the effects of varying PEF parameters (e.g., pulse duration, frequency, and field strength) on protoscolex mortality can provide valuable insights into optimizing this treatment strategy. Additionally, evaluating the combined approach in vivo will be essential for translating these findings into clinical applications. In the third group, ALB demonstrated complete efficacy against protoscoleces, achieving 100% mortality within 60 minutes of exposure. This finding underscores ALB's role as a highly effective agent against protoscoleces. However, evidence from randomized clinical trials indicates that benzimidazole derivatives (e.g., ALB and mebendazole) are insufficient to completely eradicate CE. This discrepancy between our findings and those of clinical trials may be explained by three factors. First, the structural characteristics of the cyst wall significantly differ from those of free protoscoleces. Second, the concentration of ALB used in this study (100 µg/mL) is not an oral or systemic therapeutic concentration. This concentration was directly injected into the involved tissue during electroporation solely to eliminate protoscoleces at the local site and was washed away after 60 minutes; therefore, no systemic exposure to such a high concentration occurs in the body. Accordingly, comparing this local, transient, and washable concentration with the achievable plasma levels of oral ALB (1–2 µg/mL) is not methodologically appropriate. Third, the protective barrier of the cyst wall prevents adequate penetration of benzimidazoles into the cyst, whereas free protoscoleces lack this barrier and are directly exposed to the drug (51). Thus, combining pharmacological therapy with other approaches (e.g., surgery and complementary interventions) can lead to more effective and sustained clinical outcomes. Eventually, evidence from multiple studies reveals that the combination of ALB therapy and surgical intervention sharply reduces the risk of recurrence compared to surgery alone (9, 52).

Conclusion

Our findings demonstrated that PEF significantly increases

the mortality of protoscoleces of *Echinococcus granulosus*, with mortality rates rising as pulse intensity and exposure time increase. Additionally, the combination of PEF with ALB could further enhance protoscolex mortality, showing higher efficacy compared to single treatments. Overall, these findings underline the importance of combining physical and chemical therapies to improve the protoscolicidal effect. These findings present preliminary proof-of-concept for the synergistic effect of PEF and ALB; however, clinical application remains premature and requires extensive research. Hence, further investigations are warranted to optimize PEF parameters and evaluate the combined treatment in vivo in order to better understand its mechanisms and facilitate its translation into clinical practices for the effective management of CE. Finally, future researchers must prioritize (a) experiments using clinically relevant ALB concentrations, (b) ex vivo studies on whole, isolated cysts, and (c) in vivo validation in appropriate animal models, respectively.

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Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical Approval

Ethical approval for this study was issued by the Ethics Committee of Baqiyatallah University of Medical Sciences: IR.BMSU. BLC.1402.038.

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