

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of Echinococcus Granulosus Protoscolices

Afrah A. Sadek

I-Karkh III, Baghdad Education Directorate, Baghdad, Iraq

ABSTRACT: This study evaluated the effect of some pharmacological agents on the in vitro survival of protoscolices, which were obtained from sheep naturally infected with the metacestode stage of *Echinococcus granulosus*. Oxfendazole (OFZ) at a dose of 0.05 mg/ml was tested singly and then combined with Praziquantel (PZQ) at 4 mg/ml and Albendazole (ABZ) at 1 mg/ml by this study. Amongst all these drugs, OFZ revealed maximum lethality among them. Just within five minutes of exposure, when applied alone, it produced an 85.63% mortality rate compared to 17.21% for OFZ+ABZ, 15.10% in the case of OFZ+PZQ, and only 13.09% for ABZ+PZQ—mortality rates rising up to 99.04%, 71.63%, and 40.19% as well as 31.09%, respectively after only half-an-hour exposure time recorded; within thirty-five minutes one hundred percent mortality was achieved with OFZ used singly while other combinations took about forty minutes two hours and three hours. These results consolidate the potential OFZ as a strong protoscolicidal agent in the future possible therapy against hydatid disease. It can be used after laboratory testing for its side effects on lab models of hydatid disease.

KEYWORDS: *Echinococcus granulosus*, Oxfendazole, In vitro analysis, Antiparasitic agents

INTRODUCTION

The basic treatment of hydatid cysts in humans is surgical; however, the surgery might lead to a spillage of hydatid fluid and then secondary cysts might develop (Khuroo et al., 1997). Surgical operation cannot be applied where the patient has an underlying condition or anatomically sensitive locations of cysts such as in the brain, heart, and spinal column which are difficult to access. Even if found applicable, surgical treatment has its own complications that can lead to side effects that are regarded as very serious (Alyousif et al., 2021).

Toward the efforts in the non-invasive alternative treatments, researchers have tried using antiparasitic drugs. Within this, Albendazole (ABZ) has been widely applied as adjunct postoperative therapy and in cases where surgery is not applicable to administer—as in cystic and alveolar echinococcosis—pathologies (Filice et al., 1997; Blanton et al., 1998). ABZ is absorbed from the intestinal tract and metabolized mainly to active components, such as albendazole sulphoxide (ABZSO) that may exert their effects by penetrating the germinal layer of cysts and reaching protoscolices (Cotting et al., 1990; Morris et al., 1987; Nasr et al., 2014). The drug disrupts microtubule formation by preventing β -tubulin polymerization—it attacks and kills parasite hence therapeutically promising (Dayan, 2003). Another active compound is praziquantel (PZQ), a broad-spectrum isoquinoline derivative highly effective against cestode infections that include *Echinococcus granulosus*. PZQ is well-tolerated, absorbed well following oral administration, and has a relatively short half-life of between 1 and 2 hours. The use of PZQ in combination with ABZ increases the metabolism of ABZ, consequently increasing the concentrations of its active metabolite, ABZSO by approximately 4.5 times (Urrea-Paris et al., 2000; Razmi et al., 2009). In addition to medical therapy, surgical intervention is often required for successful treatment and management of complications arising from cystic echinococcosis[5]. Medical therapy is indicated in disseminated or inoperable disease and includes albendazole (ABZ) administered at a dosage of 10–15 mg/kg daily over one or two months, repeated in cycles depending on clinical response (Taylor & Morris, 1989; Palomares et al., 2004). Some other studies outlined that this pairing reduces the size of the cyst while causing damage to internal layers within it (Nasr et al., 2014). OFZ as fenbendazole sulfoxide would circulate longer systemically than ABZ does because Gavidia et al. found that its maximum circulation time was up to 144 hours compared to less than 60 hours for OFZ versus ABZ (Gavidia et al., 2009). It may invade the cyst wall and cause degeneration of tissues, calcification, and death of protoscolices. OFZ acts by inhibiting the uptake of glucose through binding to tubulin and interfering with microtubule assembly whereby energy depletion is achieved by inhibition of ATP production in the mitochondria. This drug has broad activity, is cheap, and has long activity thus making it become a hopeful antiparasitic drug against human tapeworm infection. This study will focus on testing short-term efficacy on

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of Echinococcus Granulosus Protoscolices

early-stage larvae (protoscolices) of *E. granulosus* by applying minimal concentrations-so as to keep cytotoxicity low-of selected drugs individually as well as in combination. The aim is to find treatment options that are safer as well as more effective.

MATERIALS AND METHODS

Hydatid cysts were collected from slaughtered sheep in Al-Shu'ula district, Baghdad. Cysts were carried in a cold box to the laboratory for protoscolex separation from the germinal membrane according to the process described by Dueger (1999). The viability of protoscolices in 0.1% eosin solution was analyzed; live protoscolices appeared green while dead ones became red, and this staining method has again followed the eosin exclusion technique described by Smyth (1985). Protoscolices were kept in preservation within a solution of Krebs-Ringer buffer mixed with hydatid cyst fluid at 1:4.

A vibrator homogenized 1 ml of this solution containing an unspecified number of protoscolices. Then, 10 µl from this suspension was mixed with an equal amount of eosin dye and observed under a microscope. The entire experiment has been repeated three times. OFZ at the concentration of 0.05 mg/ml, ABZ at 1.0 mg/ml, and PZQ at 4.0 mg/ml were used in the study- their preparations were all made using physiological saline of a concentration of 0.85%. Based on the protocol described by Wangoo et al., (1989), 100 µl from each drug solution was added to separate test tubes followed by the addition of 100 µl protoscolices suspension into each tube. The samples were then incubated at a temperature of 37°C and viability checked between time intervals ranging from as short as only five minutes up to thirty-five minutes. < 100 protoscolices were counted for each sample at 10X under a light microscope to calculate viability percentages (Plate 3). SPSS version 25 was used to carry out the statistical analysis. One-way ANOVA (Fisher's test) was used to check for significance, taking P-values less than 0.05 as the level of statistical significance..

RESULTS

Tables 1–4 present results that show all drug formulas tried OFZ, OFZ+PZQ, OFZ+ABZ, and ABZ+PZQ significantly diminished protoscolex viability relative to the control group. In that group, 98.79% of protoscolices remained alive from the start to the end of the experiment. Results were statistically significant ($P < 0.05$). In figures after only a five-minute exposure, OFZ alone produced striking mortality of 85.63%. The other combinations revealed much lower killing percentages: ABZ+PZQ at 17.21%, OFZ+ABZ at 15.10%, and OFZ+PZQ at 13.44%. They increased after a half-hour exposure to rates of 99.04% for OFZ, 71.63% for ABZ+PZQ, 40.19% for OFZ+ABZ, and 31.09% for OFZ+Pzq. Complete mortality (100%) is reached in shortest time with OFZa t35 minutes; for its combination with Pzq it occurs at40 minutes; that with AB.z achieved total killing attwo hours while ab z pz q requires three hours before full lethality is achieved.

Table 1. In Vitro Assessment of OFZ Efficacy on Protoscolices Mortality Over Time

Mean ± S.E. Killing Rate (%)	Live Protoscolices	Dead Protoscolices	Total Protoscolices Count	Exposure Time (min)
85.85 ± 0.58	75	455	530	5
90.88 ± 0.30	62	618	680	10
91.96 ± 0.15	46	516	562	15
95.49 ± 0.48	22	466	488	20
97.77 ± 0.12	13	569	582	25
99.06 ± 0.03	9	951	960	30
100 ± 0.00	0	870	870	35
1.12 ± 0.00	2472	28	2500	Control

Table 2. Combined OFZ and PZQ Treatment: In Vitro Impact on Protoscolices Survival

Mean ± S.E. Mortality Rate (%)	Live Protoscolices	Dead Protoscolices	Total Protoscolices Count	Exposure Time (min)
17.33 ± 0.03	1670	350	2020	5
29.15 ± 0.30	1750	720	2470	10
43.38 ± 0.25	1070	820	1890	15
52.81 ± 1.10	1175	1315	2490	20
64.04 ± 1.20	935	1665	2600	25
72.11 ± 0.70	530	1370	1900	30
98.02 ± 0.06	40	1980	2020	35
1.12 ± 0.00	2472	28	2500	Control

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of Echinococcus Granulosus Protoscolices

Table 3. In Vitro Assessment of Protoscolicidal Activity of OFZ Combined with ABZ

% Killing Rate (Mean ± S.E.)	Protoscolices Alive	Protoscolices Killed	Total Count of Protoscolices	Exposure Duration (min)
15.14 ± 0.03	1905	340	2245	5
18.47 ± 0.06	960	220	1180	10
20.91 ± 0.17	1455	385	1840	15
28.32 ± 0.37	1215	480	1695	20
33.26 ± 0.31	1675	835	2510	25
40.32 ± 0.13	1095	740	1835	30
42.47 ± 0.15	1185	875	2060	35
1.12 ± 0.00	2472	28	2500	Control

Table 4. In Vitro Evaluation of the Combined Effect of ABZ and PZQ on Protoscolices Viability

Killing Percentage (Mean ± S.E.)	Number Surviving	Number Killed	Total Protoscolices Count	Exposure Time (min)
13.49 ± 0.12	1315	205	1520	5
15.45 ± 0.11	1450	265	1715	10
17.62 ± 0.15	1918	410	2328	15
18.68 ± 0.14	1225	285	1510	20
20.46 ± 0.09	1710	440	2150	25
31.09 ± 0.26	1758	342	1100	30
35.69 ± 0.23	1257	698	1955	35
1.13 ± 0.00	2452	28	2480	Control



Plate 1. Liver of a Sheep Infected with Hydatid Cysts

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of Echinococcus Granulosus Protoscolices



Plate 2. Germinal Layer of a Hydatid Cyst



Plate 3. Microscopic Observation of Stained Protoscolices

DISCUSSION

A most important factor in evaluating the efficacy of chemical agents used in the hydatid disease treatment is their potential to penetrate and react with the internal structure of the parasite. Several antiparasitic drugs have been reported as partially useful therapeutically in humans and animals. This has been validated for a relatively mild and early stage of the disease (WHO, 1914). With so much medical and economic antecedence attached to echinococcosis, varied strategies find favor towards its transmission minimization, especially among intermediate hosts—livestock and humans. The present study maintained protoscolices with a nutrient-rich preservative medium composed of Krebs-Ringer's solution plus hydatid cyst fluid at a 1: 4 ratio that closely simulated the physiological environment of the parasite (Smyth & Barrett, 1980). The viability of protoscolices in all groups was found to be 98.79% at the beginning of this experiment. Eosin dye was used for staining as a differentiation indicator between live and dead protoscolices. The dye enters the disrupted membranes of the cells and colors the non-viable cells red; viable protoscolices do not take up the dye, hence appear green under a microscope (Erzurumlu et al., 1995). In summary, benzimidazole derivatives have long been recognized as among the most effective chemotherapeutic agents against cystic echinococcosis in animals and humans. This paper concentrates on ABZ which presently is used mainly as a prophylactic treatment in post-surgery applications or where surgery cannot remove all the cysts (Chrieki, 2002). Once metabolized into albendazole sulphoxide (ABZSO), it will be able to penetrate the germinal layer and reach protoscolices to exercise parasitocidal action (Morris et al., 1987). Existing research revealed that neither ABZ nor praziquantel (PZQ) has much effect on ex vivo protoscolices individually but seem to demonstrate improved therapeutic results when used in combination (Urrea-Paris et al., 2000). The drug combinations may also remain active for a longer period inside host tissues which increases exposure time for protoscolices to the active compounds and enhances their parasitocidal potential (Hameed et al., 2010). Experiments of co-administration of ABZ and PZQ in mice hydatid-infected with cysts achieved significantly better results as compared to monotherapies (Pérez-Molina et al., 2011). Besides, combinations like ABZ with metronidazole (MTZ) have reflected on reducing cyst size and causing damage to both the laminated and germinal layers of hydatid cysts (Al-Obaidi et al., 2008). Similarly, the co-administration of ABZ with its metabolite ABZSO produces cytological changes such as vacuolation and lipid droplet accumulation which would disturb parasite homeostasis (Pérez-Serrano et al., 1994, 2001). Ultrastructural analysis further supported indications regarding damage effects

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of *Echinococcus Granulosus* Protoscolices

towards the germinal layer, whereby even more distinct cytoplasmic protrusions and membrane damages were observed under electron microscopy. OFZ, OFZ+PZQ, OFZ+ABZ, and ABZ+PZQ were used in this study for protoscolicidal effects at a mortality rate after 35 min exposure time among them at rates of 100%, 98.08%, 42.33%, and 35.60% respectively which are seen as Tables 1-4. These agents are believed to work by disrupting cytoskeletal elements, particularly β -tubulin, leading to inhibited formation of microtubules. Since this pathway is now known as the primary one through which benzimidazoles exert their action, disruptions in glucose uptake and glycogen depletion lead to cellular organelle damage; especially the endoplasmic reticulum and mitochondria of the cell ultimately leading to cell death by autolysis (Ingold et al., 1999; Walker et al., 2004). Livestock naturally infected with hydatid cysts have also responded positively from combination therapy treatments. For example, OFZ combined with nitazoxanide (NTZ) was also found effective and safe since no adverse effects were noticed on treated animals (Kern, 2003). High protoscolicidal activity by OFZ could most probably be due to its chemical structure wherein nitrogen atoms may create bonds with cellular proteins hence disturbing the viability of the parasite. Benzimidazole compounds are very active against *E. granulosus* protoscolices because they have a reactive hydrogen at position 1. (Gavidia et al., 2010) It is believed that these agents reduce enzymatic activity. They go through biotransformation into cytotoxic metabolites that can damage parasite DNA. (El-Harti et al., 2014) This adds more parasiticidal effect to the already existing effects. This study will go a long way to prove the fact that OFZ, either alone or in combination with other agents has high potency against hydatid disease. In vivo studies are hereby further advocated in order to assess the long-term safety as well as efficacy clinically and in veterinary settings.

CONCLUSION

The study proves that all tested drug combinations manifested different degrees of protoscolicidal activities in vitro. OFZ alone attained the highest efficacy by recording protoscolex mortality at 100% within 35 minutes. Other powerful effects were brought out by OFZ+PZQ, while ABZ+PZQ was the least effective. This finding goes a long way to empirically support all hydatid cyst management enhancement treatments based on OFZ.

REFERENCES

- 1) Al-Obaidi, H.S.; Salman, A.A. & Arziak, Z. (2008). Epidemiology of hydatid cyst with experimental trial of the anti-parasitic activity of ivermectin in compare to other common antiparasitic drug, Tikrit Med. J., 14(1): 1-11.
- 2) Alyousif, M.S.; Al-Abodi, H.R.; Almohammed, H.; Alanazi, A.D.; Mahmoudvand, H.; Shalamzari, M.H.; Salimikia, I. (2021). Chemical composition, apoptotic activity, and antiparasitic effects of *Ferula macrecolea* essential oil against *Echinococcus granulosus* protoscoleces. *Molecules*, 26, 888: 1-10. DOI:10.3390/molecules26040888.
- 3) Blanton, R.E.; Wachira, T.M.; Zeyhle, E.E.; Njoroge, E.M.; Magambo, J.K. & Schantz, P.M. (1998). Oxfendazole treatment for cystic hydatid disease in naturally infected animals. *Antimicrob. Agents Chemother.*, 42(3): 601-605.
- 4) Chai, J.-Y. (2013). Paraziquantel treatment in trematode and cestode infections: An update. *Infect. Chemother.*, 45(1): 32-43. DOI:10.3947/ic.2013.45.1.32.
- 5) Chrieki, M. (2002). Echinococcosis: An emerging parasite in the immigrant population. *Amer. Fam. Physician*, 66(5): 817-820.
- 6) Cotting, J.; Zeugin, T.; Steiger, U. & Reichen, J. (1990). Albendazole kinetics in patients with echinococcosis: Delayed absorption and impaired elimination in cholestasis. *Eur. J. Clin. Pharmacol.*, 38: 605-608. DOI:10.1007/BF00278590.
- 7) Dayan, A.D. (2003). Albendazole, mebendazole and praziquantel: Review of non-clinical toxicity and pharmacokinetics. *Acta Trop.*, 86(2-3): 141-159.
- 8) Dueger, E.L.; Moro, P.L. & Gilman, R.H. (1999). Oxfendazole treatment of sheep with naturally acquired hydatid disease. *Antimicrob. Agents Chemother.*, 43(9): 2263-2267. DOI: 10.1128/AAC.43.9.2263.
- 9) El-Harti, J.; Ansar, M. & Taoufik, J. (2014). Albendazole and its analogues. *Int. J. Pharm. Sci. Res.*, 5(3): 102-107.
- 10) Erzurumlu, K.; Özdemir, M.; Mihmanli, M. & Cevikbas, U. (1995). The effect of intraoperative mebendazole-albendazole applications on the hepatobiliary system. *Eur. Surg. Res.*, 27(5): 340-345.
- 11) Filice, C.; Brunetti, E.; D'Andrea, F. & Filice, G. (1997). Minimal invasive treatment for hydatid cysts: PAIR (puncture aspiration, injection, reaspiration) - state of the arte. *WHO/CTD/STP/97-103*.
- 12) Gavidia, C.M.; Gonzales, A.E.; Barron, E.A.; Ninaquispe, B.; Liasomas, M.; Verastegui, M.R.; Robinson, C. & Gilman, R.H. (2010). Evaluation of oxfendazole praziquantel and albendazole against cystic echinococcosis: A randomized clinical trial in naturally infected sheep, *Plos Negl. Trop. Dis.*, 4: e616. DOI:10.1371/journal.pntd.0000616.
- 13) Gavidia, C.M.; Gonzales, A.E.; Lopera, L.; Jayashi, C.; Angelats, R.; Barron, E.A.; Ninaquispe, B.; Villarreal, L.; Garcia, H.H.; Verastegui, M.R. & Gilman, R.H. (2009). Evaluation of nitazoxanide and oxfendazole efficacy against cystic echinococcosis in naturally infected sheep. *Am. J. Trop. Med. Hyg.*, 80(3): 367-372.

Evaluation of in Vitro Effects of Selected Pharmaceuticals on the Survival of *Echinococcus Granulosus* Protoscolices

- 14) Hameed, N.M.; Al-Tae, A.A. & Hamada, T.A. (2010). In vitro evaluation of the activity of *Aspergillus niger* extract on the viability of protoscolices of hydatid disease, Tikrit Med. J., 16(2): 156-168.
- 15) Ingold, K.; Bigler, P.; Thomann, W.; Gavaliero, T.; Gottstein, B. & Hemphill, A. (1999). Efficacies of albendazole sulfoxide and albendazole sulfone against in vitro-cultivated *Echinococcus multilocularis* metacestodes. Antimicrob. Agents Chemother., 43(5): 1052-1061. DOI:10.1128/AAC.43.5.1052.
- 16) Kern, P. (2003). *Echinococcus granulosus* infection: Clinical presentation, medical treatment and outcome. Langenbecks Arch. Surg., 388: 413-420. DOI:10.1007/s00423-003-0418-y388: 413-20.
- 17) Khuroo, M.S.; Wani, N.A.; Javid, G.; Khan, B.A.; Yattoo, G.N.; Shah, A.H. & Jeelani, S.G. (1997). Percutaneous drainage compared with surgery for hepatic cysts. New Engl. J. Med., 337(13): 881-887. DOI: 10.1056/NEJM199709253371303.
- 18) Morris, D.L.; Chinnery, J.B.; Georgiou, G.; Stamatakis, G. & Golematas, B. (1987). Penetration of albendazole sulphoxide into hydatid cysts. Gut, 28: 75-80.
- 19) Nasr, W.T.; Saadeh, H.A.; Mubarak, M.S. & Abdel-Hafez, S.K. (2014). In vitro activity of novel metronidazole derivatives on larval stage of *Echinococcus granulosus*. Jordan J. Biol. Sci., 7(3): 187-194.
- 20) Njoroge, E.; Mbithi, P.; Wachira, T.; Gathuma, J.; Gathura, P.; Maitho, T.E.; Magambo, J. & Zeyhle, E. (2005). Comparative study of albendazole and oxfendazole in the treatment of cystic echinococcosis in sheep and goats. Int. J. Appl. Res. Vet. Med., 3(2): 97-101.
- 21) Palomares, F.; Palencia, G.; Pérez, R.; González-Esquivel, D.; Castro, N. & Cook, H.J. (2004). In vitro effects of albendazole sulfoxide and praziquantel against *Taenia solium* and *Taenia crassiceps* cysts. Antimicrob. Agents Chemother., 48(6): 2302-2304. DOI:10.1128/AAC.48.6.2302-2304.2004.
- 22) Pérez-Molina, J.; Díaz-Menéndez, M.; Gallego, J.I.; Norman, F.; Monge-Maillo, B.; Ayala, A.P. & López-Vélez, R. (2011). Evaluation of nitazoxanide for the treatment of disseminated cystic echinococcosis: Report of five cases and literature review. Am. J. Trop. Med. Hyg., 84(2): 351-356. DOI:10.4269/ajtmh.2011.10-0513.
- 23) Pérez-Serrano, J.; Casado, N.; Denegri, G. & Rodríguez-Caabeiro, F. (1994). The effects of albendazole and albendazole sulphoxide combination-therapy on *Echinococcus granulosus* in vitro. Int. J. Parasitol., 24(2): 219-224. DOI: 10.1016/0020-7519(94)90029-9.
- 24) Pérez-Serrano, J.; Martínez, J.; Denegri, G.; Casado, N.; Bodega, G. & Rodríguez-Caabeiro, F. (2001). In vitro effect of albendazole and albendazole-sulphoxide on 70 and 60 KDa heat shock proteins in *Echinococcus granulosus* protoscolices. Rev. Ibérica Parasitol., 61: 67-71.
- 25) Razmi, G.R.; Hashemi Tabar, G.R. & Maleki, M. (2009). In vitro effect of albendazole and mebendazole on hydatid cyst of mice. Arch. Razi. Inst., 64(1): 45-50.
- 26) Schwabe, C.W.; Hadidian, L. & Koussa, M. (1963). Host-parasite relationships in echinococcosis scolices. IX. In vitro survival of hydatid scolices and the effects of drugs upon scolex respiration. Am. J. Trop. Med. Hyg., 12: 338-345.
- 27) Smyth, J.D. (1985). In vitro culture of *Echinococcus* spp. Proc. 13th Int. Cong. Hydatidol., Madrid: 84-89.
- 28) Smyth, J.D. & Barret, N.J. (1980). Procedures for testing the viability of human hydatid cysts following surgical removal, especially after chemotherapy. Trans. Roy. Soc. Trop. Med. Hyg., 74: 849-852.
- 29) Taylor, D.H. & Morris, D.L. (1989). Combination chemotherapy is more effective in postspillage prophylaxis for hydatid disease than either albendazole or praziquantel alone. Br. J. Surg., 76(9): 954. DOI:10.1002/bjs.1800760927.
- 30) Urrea-Paris, M.A.; Moreno, M.J.; Casado, N. & Rodríguez-Caabeiro, F. (2000). In vitro effect of praziquantel and albendazole combination therapy on the larval stage of *Echinococcus granulosus*. Parasitol. Res., 86(12): 957-964. DOI:10.1007/pl00008526.
- 31) Walker, M.; Bazz, A.; Dematteis, S.; Stettler, M.; Gottstein, B.; Schaller, J. & Hemphill, A. (2004). Isolation and characterization of a secretory component of *Echinococcus multilocularis* metacestodes potentially involved in modulating the host parasite interface. Infect. Immun., 72(1): 527-536. DOI:10.1128/IAI.72.1.527-536.2004.
- 32) Wangoo, A.; Ganguly, N.K. & Mahajan, R.C. (1989). Phagocytic function monocytes in murine model of *Echinococcus granulosus* of human origin. Indian J. Med. Res., 89: 40-42.
- 33) WHO (2014). Oxfendazole: Safety summary for veterinary use on dogs, cats, cattle, sheep, goats, swine, horses and poultry. Poisoning, intoxication, overdose, antidote: 1-5.