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Effect of Some Drugs on the Viability of the Protoscolices of *Echinococcus granulosus* (Batsch, 1876) in Vitro

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Abstract: This study was conducted to investigate the effect of a number of drugs on the viability of primary protoscolices isolated from sheep infected with the larval stage of *E. granulosus*. These drugs included Oxfendazole (OFZ) at a concentration of 0.05 mg/ml, mixed with Praziquantel (PZQ) at a concentration of 4 mg/ml and Albendazole (ABZ) at a concentration of 1 mg/ml. The results showed that OFZ was the most effective in influencing the viability of the protoscolices. The percentage of killing after five minutes of treatment was 85.63% and it was 17.21%, 15.10% and 13.09% for OFZ+ABZ, OFZ+PZQ and ABZ+PZQ, respectively. After 30 minutes, the killing percentage was 99.04%, 71.63%, 40.19% and 31.09%, respectively. The percentage of killing reached 100% after 35 minutes, 40 minutes, 2 hours and 3 hours, respectively. The results confirm the use of OFZ as a lethal drug, or a viability-suppressant for protoscolices in the future, after conducting more researches to know the side effects of this drug in laboratory animals for access to the best treatment to those infected with hydatid cysts.

Keywords: *Echinococcus granulosus*, Oxfendazole, In vitro, Chemical drug

Introduction

Surgical treatment of hydatid cysts is the first line of treatment in humans, despite of the possibility of leakage of hydatid cystic fluid, that leads to the occurrence of secondary hydatid cysts (Khuroo et al., 1997), or the patient is not medically qualified for surgery, or the cyst may fall in locations that are difficult to deal with surgically, such as cerebral hydatid cysts or attached to the heart or spinal cysts. However, serious risks during hydatid cyst surgery are not completely safe and have various adverse side effects (Alyousif et al., 2021). So, researchers were able to find alternative means to surgical treatment by using drugs that are only partially successful, including the use of ABZ (Filice et al., 1997) which is one of the effective drugs in the preventive treatment after surgical operations, and cases that cannot be treated in alveolar and cystic hydatid disease (Blanton et al., 1998). The drug ABZ is absorbed by the small intestine and metabolized into some derivatives, including Albendazole sulphoxide (ABZSO) which is involved in the effective therapeutic activity of the hydatid sacs (Cotting et al., 1990), and it has the ability to reach the germinal layer and primary protoscolices in the hydatid sac (Morris et al., 1987; Nasr et al., 2014).

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Albendazole works to prevent polymerization of B-tubulin to the microtubules, leading to the destruction of the parasite, which gave promising indications to control the disease (Dayan, 2003).

The Praziquantel (PZQ) is a compound derived from isoquinline. It has an effective impact on several types of tapeworms, including *Echinococcus granulosus* (Batsch, 1876). PZQ is rapidly absorbed by the body when taken orally and is well tolerated, with an average half-life of 1-2 hours (Chai, 2013).

Research has shown that ABZ has a slower killing effect on the protozoans if used alone, compared to when used with PZQ (Urrea-Paris et al., 2000; Razmi et al., 2009). Since PZQ works to increase the metabolism of ABZ to ABZSO by 4.5 times. The use of both drugs together has an effective impact on the secondary hydatid cyst in the treated animals compared to the treatment regimen alone (Taylor & Morris, 1989; Palomares et al., 2004), as the hydatid cysts were exposed to ABZ and metronidazole (MTZ-S) causes a reduction in the size of the cysts as well as damage to both the lamellated and germinal layers of the cyst (Nasr et al., 2014).

One of the veterinary drugs used to treat many tapeworms, including hydatid cysts in sheep, cows and pigs, is Oxfendazole (OFZ). It is Fenbendazole methyl ester sulfoxide (Phenylsulfinyl)-1-H-benzimidazole-2yl Carbamic acid. It is absorbed by the intestine and present in the plasma for more than 144 hours after treatment compared to ABZ which is present for a period of 60 hours after treatment (Gavidia et al., 2009). The OFZ has the ability to penetrate the cyst wall and kill the living tissue of the hydatid cyst and the separation of the internal endocyst sacs. The cysts are calcified and the primary proto-heads are killed by 3.93% in sheep treated experimentally (Njoroge et al., 2005).

The mechanism of OFZ is the inhibition of microtubule polymerization through its association with tubulin. It also prevents glucose uptake, which leads to paralysis and death of the parasite, and it prevents the generation of energy in the form of adenosine triphosphate (ATP) of the mitochondria. Therefore, it is considered as one of the useful and inexpensive drugs for the treatment of tapeworm infection in humans (WHO, 2014).

The current study aimed to investigate a drug that is effective on the primary markers of the larval stage of *E. granulosus* within a short period of time and with a lowest possible concentration to reduce its toxic effect on the cells of the body, in the hope of obtaining effective methods of treatment.

Materials and Methods

Hydatid cyst samples were collected from infected sheep slaughtered in Al-Shu'ula City in Baghdad (Plate 1), and transferred in a refrigerated container to the laboratory to isolate the protoscolices from the germinal membrane (Plate 2) according to Dueger et al. (1999). An amount of percentage of the viability of protoscolices according to penetration of eosin stain (0.1%) that appeared in bright green color was calculated compared to the dead protoscolices that were stained in red (Plate 3) after each exposure period. The viability percentages was determined

by calculating the percentage of dead and live protoscolices by means of an eosin exclusion experiment according to Smyth (1985).

The protoscolices were placed in the preservative medium (Krebs-Ringer solution + hydatid cyst fluid in a ratio of 1:4) according to Smyth & Barret (1980). One ml of the solution containing an unknown number of protoscolices was taken with the preserving medium and stirred with a vibrator. Ten microliters were taken from the proprietary suspension (primary capillaries + preservative medium) and mixed with 10 µl of eosin dye and examined under the microscope. The rate of three replicates was adopted (Schwabe et al., 1963).

OFZ (concentrated 0.05mg/ml), 2- ABZ at a concentration of 1.0 mg/ml, 3- PZQ at a concentration of 4.0 mg/ml. These concentrations were prepared in physiological solution (normal saline 0.85%). According to Wangoo et al. (1989), 100 microliters of the concentrations under study were placed in each test tube, 100 microliters of the primary capillary suspension were added, and the tubes were incubated at a temperature of 37 °C. After 5-35 minutes, the viability of the protoscolices was calculated. Then the percentage of viable protoscolices (viability rate) was determined by counting a minimum of 100 protoscolices (as a ratio of number of viable protoscolices to total protoscolices). The survival rate of the protoscolices was taken after examining them under the microscope at X10 (Plate 3).

One-Way ANOVA (Fisher test) and SPSS program (V.15) were used to analyze the results, and $P<0.05$ was considered as statistically significant value.

Results

The results (Tables 1-4) showed that each of the used drugs (OFZ, OFZ+PZQ, OFZ+ABZ and ABZ+PZQ) at the used concentrations were lethal substances for the primary targets and that the percentage of killing increased with time to varying degrees compared to the control group, in which 98.79% remained viable for the duration of the experiment. The results of the statistical analysis confirmed that there were significant differences at the probability level of 5 ($P<0.0$). As the percentages of killing when treating the primary principals with OFZ, ABZ+PZQ, OFZ+ABZ and OFZ+PZQ for five minutes were 85.63%, 17.21%, 15.10% and 13.44%, respectively. After 30 minutes, the percentage of killing were 99.04%, 71.63%, 40.19% and 31.09%, respectively. The 100% percentage of killing for OFZ was 35 minutes, for OFZ+ PZQ was 40 minutes, and for OFZ+ABZ was 2 hours, while that for ABZ+PZQ was three hours.

Table 1: Effect of OFZ on protoscolices viability in vitro.

S.E total percentage (%) of killing	Number of viable protoscolices	Number of killed protoscolices	Total number of protoscolices	Time (minute)
85.63±0.61	75	447	522	5
90.33±0.33	63	610	673	10
91.88±0.12	55	510	555	15
95.65±0.56	21	460	481	20
97.56±0.17	14	563	577	25
99.04±0.04	9	944	953	30
100±0	0	873	873	35
1.2±0	2450	30	2480	Control

Table 2: effect of OFZ+PZQ on protoscolices viability in vitro.

S.E total percentage (%) of killing	Number of viable protoscolices	Number of killed protoscolices	Total number of protoscolices	Time (minute)
17.21±0.02	1669	347	2016	5
29.03±0.35	1752	715	2467	10
43.09±0.27	1072	810	1882	15
52.66±1.16	1180	1300	2480	20
63.88±1.27	940	1650	2590	25
71.63±0.76	535	1360	1895	30
98.08±0.07	39	1977	2016	35
1.2±0	2450	30	2480	Control

Table 3: Effect of OFZ+ABZ on protoscolices viability in vitro.

S.E total percentage (%) of killing	Number of viable protoscolices	Number of killed protoscolices	Total number of protoscolices	Time (minute)
15.10±0.04	1899	338	2237	5
18.30±0.07	960	215	1175	10
20.79±0.18	1452	380	1832	15
28.15±0.39	1212	475	1687	20
33.19±0.33	1671	830	2501	25
40.19±0.14	1093	735	1828	30
42.33±0.16	1186	870	2056	35
1.2±0	2450	30	2480	Control

Table 3: Effect of ABZ+ PZQ on protoscolices viability in vitro.

S.E total percentage (%) of killing	Number of viable protoscolices	Number of killed protoscolices	Total number of protoscolices	Time (minute)
13.44±0.13	1312	203	1515	5
15.23±0.12	1450	260	1710	10
17.47±0.16	1916	405	2321	15
18.56±0.15	1228	280	1508	20
20.32±0.10	1708	435	2143	25
31.09±0.27	1708	340	1097	30
35.60±0.24	1255	695	1950	35
1.2±0	2450	30	2480	Control

Plate 1: Liver infected with hydatid cysts.

Plate 2: Hydatid cyst germinal membrane.

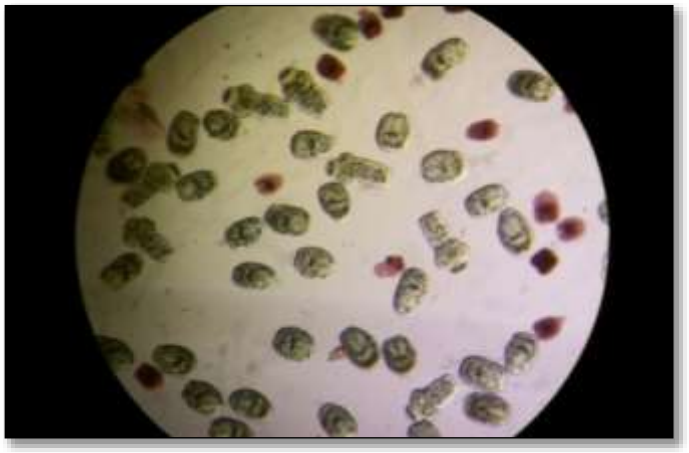

Plate 3: Microscopic view showing the viable (bright green) and dead protoscolices (red).

Discussion

One of the important criteria for evaluating the efficiency of any chemical drug used in the treatment of hydatid cysts is the access of that drug to the parasite's structure. Generally, these drugs have partial effects in treating the disease in humans and animals, depending on the development of the disease (WHO, 1914).

Due to the importance and seriousness of this disease, many attempts and solutions have been taken to reduce or limit the infection and its spread among intermediate hosts including humans. In this experiment, the preservative medium (Krebs-Ringer solution + hydatid cyst fluid at a ratio of 1:4) was adopted to maintain the protoscolices. It is a complex solution that contain different materials commensurate with the need of protoscolices of nutrients (Smyth & Barret, 1980).

The viability percentage of protoscolices at the beginning of the experiment at time zero was 98.79% for all drugs used. As the penetration of eosin pigment is a physical process, so any physiological defect occurs for any reason, permeability increases and hence it allows the dye to enter, while the live protoscolices continue to display their usual green color (Erzurumlu et al., 1995).

Drugs from benzimidazole derivatives, which are chemicals their effectiveness against animal and human cystic echinococcosis have been proven by many researchers (Chrieki, 2002).

Albendazole is used in the field for preventive treatment after surgeries, in cases that cannot be treated surgically and when it is metabolized to ABZSO which has the ability to reach the protoscolices and the germinal layer inside the hydatid sac (Morris et al., 1987).

The monotherapy of ABZ or PZQ has lower toxicity to ex *vivo* protoscolices than if it is used in combination of ABZ + PZQ (Urrea-Paris et al., 2000).

Mixing more than one drug may lead to the formation of a complex that may remain for a longer period. This means an increase in the period of exposure of the protoscolices to the drug, and thus increase its effects on the protoscolices (Hameed et al., 2010). When a mixture of ABZ + PZQ was used in treating mice

experimentally infected with hydatid cysts, it gave better results than if the two drugs were used separately (Pérez-Molina et al., 2011).

The use of ABZ and MTZ together caused a reduction in the size of the hydatid cyst and damaged the germinal membrane with exfoliation of the laminated layer of the hydatid cyst. The possibility of using the treatment resulting from the combination of ABZ + PZQ in patients is achieved with hydatid cysts that cannot be surgically removed (Al-Obaidi et al., 2008).

The use of ABZ + ABZSO increases the gaps and the appearance of many fatty droplets and platelets, which can be considered as one of the direct effects of the mixed drug used in the chemical treatment of hydatid cysts (Pérez-Serrano et al., 1994, 2001).

It was noticed that a mixture of ABZ + ABZSO caused damage to the germinal layer, including the appearance of cytoplasmic protrusions with the appearance of many gaps upon examination by the electron microscope as stated by some researchers in treating the protoscolices with different chemical compounds (Pérez-Serrano et al., 1994, 2001).

In the current study, OFZ, OFZ + PZQ, OFZ + ABZ and ABZ + PZQ were used that killing protoscolices at rates of 100%, 98.08%, 42.33% and 35.60%, respectively after 35 minutes (Tables 1-4). The mechanism of the action of the chemical drugs depends on the interaction with the cytoskeletal protein, which is B-tubulin, and preventing its polymerization into microtubules. Pyridazine derivatives bind with the parasite tubulin and work to reduce glucose uptake, which in turn leads to depletion of glycogen stores, producing degradative changes to the endoplasmic reticulum and mitochondria in the germinal layer, the release of lysosomes and the occurrence of larval autolysis (Ingold et al., 1999; Walker et al., 2004). The use of more than one drug to treat sheep naturally infected with hydatid cysts gave encouraging results, including the use of OFZ with the NTZ. No side effects were observed in any of the treated groups (Kern, 2003).

The effective impact of OFZ in killing the protoscolices may be due to the presence of nitrogen atoms, each of which has an electron pair capable of forming bonds or competitive bonds with the parasite cell, thus forming cytotoxic compounds that bind to the protein of the protoscolex, causing its death. As the benzimidazole derivatives possess a hydrogen atom number 1, it is important against *E. granulosus* protoscolices (Gavidia et al., 2010). These substances reduce the activity of many enzymes. Killing occurs as a result of enzymes present in the parasites that work to convert this drug into a cytotoxic drug, because the toxic compound resulting from this activation process cause damage to the DNA of the parasite (El-Harti et al., 2014).

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