



A Comparison between the Effects of Albendazole and Mebendazole on the Enzymatic Activity of Excretory / Secretory Products of *Echinococcus granulosus* Protoscoleces in Vitro

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(Received 15 May 2015; accepted 10 Aug 2015)

Abstract

Background: Hydatid cysts are formed in human body can be treated clinically by surgery or drugs such as albendazole (ABZ) and mebendazole (MBZ). The purpose of this study was comparing the effects of ABZ and MBZ on glutathione-S-transferase, alkaline phosphatase and protease enzymes activities in protoscoleces of hydatid cyst.

Methods: The culture supernatants containing the parasite Excretory / Secretory (E/S) products were collected every 12 h for 72 h. The E/S products of treated samples with 1µg/ml ABZ and MBZ and the control one were collected and after centrifugation then protein concentrations were measured according to Bradford method. GST, ALP and protease activities of E/S products were assessed photometrically.

Results: The mean of GST specific activity level in treated protoscoleces with ABZ and MBZ and in control group were obtained 69.44, 132.83 and 225.47U/mg/protein/ml respectively. The mean ALP activity level in treated protoscoleces with ABZ and MBZ and in control group were detected 19.22, 22.27 and 27.85 U/mg/protein/ml respectively. The protease activity level in treated protoscoleces with ABZ and MBZ were not detected. While the mean of protease activity level in control group was 7.61U/mg/proteins. Statistical analysis showed the significant difference between protein concentrations, the specific activities of GST, ALP and protease enzymes in treated protoscoleces in comparison with control group ($P<0.05$). Also, the significant difference were seen between specific activities of GST and ALP enzymes in treated protoscoleces with ABZ in comparison with treated group with MBZ ($P<0.05$).

Conclusion: ABZ is more effective on the enzymes activities (GST and ALP) as compared with MBZ.

Keywords: Hydatid cyst protoscoleces, Albendazole, Mebandazole, Protease, Glutathione S-Transferase, Alkaline phosphatase

Introduction

Echinococcosis or cystic hydatid disease is an endemic parasitic disease in human populations in Iran and some parts of the world. It is caused by larvae stage of *Echinococcus granulosus* (1). In the life cycle of *E. granulosus*, humans sometimes play ropes as accidental intermediate host. Clinical treatment of

cysts includes albendazole (ABZ) or mebendazole (MBZ) therapy in combination with either surgical resection (2).

The role of enzymes in living organism is clear and remarkable and the worms are seriously dependent on these activities. The deficiency or in-

hibition of the enzyme activities will prevent parasite survival. Parasite components such as enzymes have specific biological functions, which are necessary for parasite survival and are supposed to have an important role in host-parasite interactions and disease progress (3). Parasite's enzymes are attractive purposes that are explored for the development of diagnostic method and vaccines. They mediate processes like tissue invasion, feeding, evasion (Escape the immune system) of host immune response etc. (4). Glutathione-S-Transferase (GST) is an enzyme, which has a significant role in the detoxification of parasite metabolites (Endogenous), host metabolites (Xenobiotics) and drugs through their conjugation to glutathione (5). GST activity in *E. granulosus* has been described in the cytosolic portion of protoscoleces obtained from sheep cysts and activated by pre-treatment of protoscoleces with GST inducers (6).

Alkaline phosphates (ALP) is an enzyme that plays an important role in dampening host immune responses and also plays a role in feeding parasites (7). Several isoenzymes of ALP have been detected in worms. Most phosphatases have been found in the absorption system of cestodes, excretory system in trematodes and intestinal cells of the nematode (8). Proteolytic enzymes of parasites have been given more attention than other enzymes, because they play a vital role in parasite survival and are involved in many fundamental physiologic processes (3). The activities of protease described in *E. granulosus* (9), has been detected in hydatid cyst fluid, cyst wall and in protoscoleces. This enzyme is responsible for breakdown of proteins in all living tissues in order to be used by the cells (10). In addition to their known role in the catabolism, they have a part in protein processing in evasion from immune system, leaving the cyst, molting of the parasites and in diagnosis, especially cysteine proteases as serological markers. Proteases have generally been identified as potential drug targets in parasites (11, 12).

The purpose of this study was to determine the effects of ABZ and MBZ on the activity of the GST, ALP and proteases in the protoscoleces of

hydatid cyst and to evaluate their inhibitory effects on enzyme activity.

Materials and Methods

Collection of Protoscoleces

Protoscoleces were obtained by aseptic puncture from fertile liver hydatid cysts of ovine origin collected from an abattoir in Rey City in Tehran (center of Iran). Protoscoleces were allowed to settle in a 50 ml Falcon tube, and then washed several times in phosphate-buffered saline (PBS pH, 7.2). Viability was determined by eosin 0.01 exclusion analysis and only protoscoleces samples with viability higher than 95% were selected for the assays (13).

MBZ used in this study was obtained from Rouzdarou Pharmaceutical Company (Iran) and ABZ was purchased from Tolide Daruhai Dami Iran Company.

Culture protoscoleces

Five culture medium [RPMI 1640 (Gibco, CET. No:K4111-500), 100U/ml of penicillin and 100µg/ml of streptomycin as 1 ml for each] containing 500µl protoscoleces and 1 µg/ml ABZ and/ MBZ [stock solution 1 mg/ml of dimethyl sulphoxide (DMSO)] were considered as test groups and 10 culture medium [five culture containing 500 µl protoscoleces with 0.6 µl DMSO, and five culture medium without DMSO] regarded as control groups and were incubated at 37 °C in 5% CO₂ (14).

Excretory / Secretory products collection

The culture supernatants containing the parasite E/S products were collected at time interval, every 12 h. At the end of each time interval, the entire 1 ml of culture medium (culture supernatants) was removed and replaced with the same volume of fresh medium (13). The medium supernatant was centrifuged at 10000×g at 4 °C. The precipitates were discarded and the total protein and enzyme activities were measured as follows:

Protein concentration measurement of E/S products

The concentrations of total proteins of E/S samples were measured by Bradford method, which

involves reacting the E/S samples with a dye that binds to protein. To measure the protein concentration, standard solutions (Bovine Serum Albumin) and E/S products were prepared and the Bradford reagent (100 mg Coomassie Brilliant Blue G-250, 50 ml 95% ethanol, 100 ml 85% phosphoric acid) was added. The absorbance of E/S products and standard solutions were measured at 595 nm after 5 min incubation at room temperature. A standard curve was prepared by using the standard solutions absorbance and the protein concentration of the samples were estimated (15, 16).

Glutathione-S- Transferase assay

In order to measure the activity of GST in E/S samples, reagent stock including potassium phosphate buffer 0.1 M, 100 mM reduced glutathione (GSH) and 100 mM 1-chloro-2,4- dinitrobenzene (CDNB) substrates were prepared in a microtube. To each test, from the mentioned mixture 1.8 ml and 200ul of ABZ and MBZ treated protoscolecis E/S sample were added and mixed well. The same method was performed for the control groups and absorbances of GST activities were measured at 340 nm for 5 min. Finally, Total GST activity (U/ml), of samples was calculated. To calculate the specific activity of GST enzyme, the rate of enzyme activity was divided by the mg protein concentration (17).

Alkaline phosphates assay

ALP activity was measured using Pars Azma ALP kit (REF=10-503). Eight hundred μ l of buffer reagent (R1) and 200 μ l of substrate reagent (R2) were poured into cuvette and mixed. Then 20 μ l of treated or control samples were added and mixed well and measured absorbance of sample for 5 min by every 1 min at 405 nm and enzyme activity were calculated according to the kit procedure.

Protease assay

Sasein solution 0.65% (6.5 mg/ml of casein in the 50 mM potassium phosphate buffer 50 mM,

pH7.5) prepared and incubated at 37 °C for 5 min, and then E/S samples were added to test tubes and were incubated 37 °C for 10 min. The reaction was stopped using trichloroacetic acid (110 mM TCA, prepared by diluting a 6.1N stock 1:55 with purified water). E/S products were added to control tubes simultaneously and incubated for 30 min at 37 °C, and then centrifuged for 5 min at 14000 \times g at 25 °C. The supernatant was poured into the test tube; 1cc sodium carbonate solution (500 mM Sodium Carbonate solution, prepared using 53 mg/ml of anhydrous sodium carbonate in purified water) and 200 μ l Folin & ciocalteus phenol reagent were added and incubated for 30 min at 37 °C. Finally, tubes were centrifuged for 5 min at 14000 \times g at 25 °C and Absorbances were measured spectrophotometrically at 660 nm. The protease activity was compared with a standard curve (Std. solution L-tyrosin 1.1 Mm, DW, Na₂CO₃, phenol) and reported as Units /ml enzyme (16). When the protease digests casein as substrate, the amino acid tyrosine is released. Folin & ciocalteus phenol reagent primarily react with free tyrosine and produce a blue color.

Statistical analysis

In order to determine the statistically significant difference between protein concentrations, GST, ALP and protease activities of E/S samples of treated and control groups, *t*-test was used. ([http://www.socscistatistics.com/tests/student test/](http://www.socscistatistics.com/tests/student%20test/)). A duplicate set of samples were taken for each test and at the end, their average was taken in to account.

Results

Protein concentration in treated and control groups of E/S products

The protein concentrations in the E/S products are shown in Table 1. The mean protein concentrations in E/S samples of protoseolecic exposed to ABZ and MB were measured 3.55 and 4.58 μ g/ ml, respectively. The mean protein concentration in the control group was 6.06 μ g / ml .

Table 1: Protein concentration of E/S products of protoscoleces treated and control groups

E/S product samples	Protein Concentrations($\mu\text{g/ml}$)		
	Control	Albendazole treated group	Mebendazole treated group
S1	5.12	2.43	3.65
S2	5.71	2.97	4.51
S3	6.41	4.89	5.81
S4	6.91	4.17	4.23
S5	6.17	3.30	4.71

$P < 0.05$; between control and treated groups

$P > 0.05$; between treated groups

GST, ALP and Protease activity in treated and control groups of E/S products

The results of GST, ALP and Protease activity are shown in Table 2. GST specific activity level of protoscoleces in ABZ group was 69.44 and in MBZ treated group was 132.82 U/mg protein/ml. GST specific activity in control group was 225.47 U/mg protein/ml. ALP specific activity in ABZ

and MBZ treated protoscoleces were estimated as 19.22 and 22.27 U/mg protein/ml, respectively. ALP specific activity of control group of E/S products was 27.85 U/mg protein/ml. Protease specific activity in ABZ and MBZ treated protoscoleces E/S products was not detected, while protease specific activity of control group E/S products calculated 7.61 U/mg protein/ml.

Table 2: GST, ALP and Protease activity in E/S products of protoscoleces treated and control groups

E/S Product	Control			Albendazole treated group			Mebendazole treated group		
	GST* specific activity (U/mg protein/ml)	ALP** specific activity (U/mg protein/ml)	Protease*** specific activity (U/mg protein/ml)	GST specific activity (U/mg protein/ml)	ALP specific activity (U/mg protein/ml)	Protease specific activity (U/ml mg protein/ml)	GST specific activity (U/mg protein/ml)	ALP specific activity (U/mg protein/ml)	Protease specific activity (U/mg protein/ml)
T1	216.66	28.28	11.81	61.00	17.96	N/D	123.12	20.91	N/D
T2	270.00	28.31	6.66	32.53	21.71	N/D	133.33	23.68	N/D
T3	226.82	26.50	6.55	93.84	18.23	N/D	145.77	22.83	N/D
T4	196.66	28.75	4.89	83.85	17.21	N/D	134.28	21.45	N/D
T5	217.20	27.43	8.12	76.00	19.91	N/D	127.64	22.50	N/D

$P < 0.005$, T=test or sample N/D= not detected

$$*GST \text{ activity} = \frac{\Delta OD/min}{0.0096} \times \frac{\text{Total volume}}{\text{Sample volume}} \times \text{dilution factor}$$

$$**ALP \text{ activity} = \Delta OD \times 2764$$

$$*** \text{ Protease activity} = \frac{(\mu\text{mole tyrosine equivalents released})}{\text{Volume of enzyme} \times \text{Time of assay} \times \text{Volume of filtrate}} \times (\text{Total volume of assay})$$

$$\text{Specific activity} = \frac{\text{Units/ml enzyme}}{\text{mg solid}}$$

Statistical analysis

Statistical analysis using *t*-test showed the significant difference between protein concentrations and specific activities of the enzymes in E/S products of protoscoleces treated with ABZ and MBZ in comparison with control group ($P < 0.05$). A sig-

nificant difference was observed between specific activity of GST and ALP enzymes in E/S products of protoscoleces treated with ABZ in comparison with the group treated with MBZ ($P < 0.05$). The protease activity was not detected in any of the both drug groups. Meanwhile there

is no significant difference between protein concentration in E/S products of protoscolecetes treated with ABZ compared with the group treated with MBZ ($P>0.05$)

Discussion

Glutathione transferases (GST) are multifunctional enzyme present in both animal and plant kingdoms. The enzymes are regarded as parts of the phase II detoxification system that catalyse glutathione (GSH) conjugation of a multitude of exogenous and endogenous toxic compounds (18). GST is one of the major detoxification system component found in helminthes, particularly at high levels in cestodes and digeneas. In the tissue extracts of parasites GST activity has been found in different ranges (19). GST activity exists in E/S products and in the surface of digenea worms such as *Schistosoma*, *Fasciola* and hookworm *Necator*. GST in E/S products act as an anti-inflammatory agent and neutral lipid peroxidation products in the mucous membranes (20, 21). Researches related to chemotherapy and immunotherapy, have identified glutathione of parasitic worms independently as a potential target for treatment (22). Proteolytic enzymes secreted by parasites and worms are well proved. These enzymes play a vital role in ensuring the parasite's life cycle. These acts include the digestion of host's tissue in order to provide food for parasites, preventing blood coagulation, facilitating entry into the host's immune system, and disrupting it (23).

ALP is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides and proteins (24). The phosphatases have been found frequently in the structure of absorption like tegument of cestodes, intestinal cell of the nematodes and excretory system of trematodes. While alkaline phosphatases have been found in adult cestode, the larval forms have a predominance of acid phosphates. This change in these enzymes may be associated with the growth and development of the reproductive system in adult worms (8). ALP as protein plays a role in immune evasion by generating the potent immunosuppressant adenosine. Since adenosine is

also an important nutrient, ALP may also play a role in parasite feeding (7).

Several studies have been carried out on characterization of hydatid cyst protoscolecetes E/S products (25, 26). In the present study, the effect of ABZ and MBZ on protein content and some enzymatic activities of E/S products were examined. Protein concentration in treated groups E/S products were less than that of the control group E/S sample. ABZ and MBZ cause degenerative changes in intestinal cells of the worm by binding to the colchicine-sensitive site of tubulin, therefore blocks its polymerization into microtubules and inhibits the cell proliferation in metaphase stage (27, 28). Based on these findings we can expect the decreasing of total protein in treated parasite culture media, which correlates our study that shows an average protein of test group is less than control group.

In this study, a significant decrease observed in the activity of GST, ALP and protease enzymes of the E/S products of treated groups in comparison with the control group. The inhibition of enzymes activity induced by MBZ and ABZ might damage the defensive system, metabolism and nutrition of the parasite. ABZ drug reduces the secretory GST enzyme (5). The inhibition of GST activity induced by anti-helmintics may contribute to passive the detoxification mechanism (29). The ABZ and MBZ cause reduction in protease enzymes activity, and effect on the parasite activities including assault, migration, feeding, and particularly survival in the host. Since protease enzymes are proteins, the reduction of their activity could be attributed to interference of benzimidazole with protein synthesis (30).

ALP is an important enzyme of helminthes parasites associated with absorption and/or digestion of food materials. It has a role in modulating the host immune response (7, 31). Higher concentrations of ALP found in the some areas of intestine and sub-cuticular layers of the worm associated with protein transport processes (32). Various commercial drugs (anthelmintic) and chemical compounds are shown to bring about changes in the activity of ALP in various soft-bodied helminth parasites like cestodes (33). When *Taenia*

solium cysts was treated with praziquantel and ABZ, ALP secretion in the culture medium have been found to be reduced (34).

Based on the results obtained in this study, the reduction or inhibition of the enzymes (GST and ALP) caused by ABZ and MBZ could lead to alteration of metabolism in helminthes parasites. Therefore, the study indicates that both anti hydatid benzimidazoles (ABZ and MBZ) have an inhibitory effect on the activity of GST, ALP and protease enzymes in E/S products of *E. granulosus* protoscoleces in vitro.

Protein concentration in E/S products of MBZ groups was slightly higher than that of the ABZ groups but this difference was not significant.

The results showed that the effect of ABZ on the activity of GST and ALP is more than MBZ and this may be due to the different structures of these drugs. In addition, the above-mentioned results might account for at least in part, difference in anti-hydatid mechanism of the both benzimidazole (5).

Conclusion

Both drugs have an inhibitory effect on the activity of GST, ALP and protease enzymes. The results of the two drugs indicated that ABZ is more effective on the enzymes activities (GST and ALP) as compared with MBZ. This may be attributed to the different structures of the two drug and might account for at least in part, difference in anti-hydatid mechanism of these benzimidazole derivation.

Acknowledgement

We would like to express our appreciation to the staffs of Soleimani slaughterhouse, Rouzdarou Pharmaceutical and Tolide Daruhai-e Dami Iran Company. Moreover, thank to Tehran University of Medical Sciences for financial support of study (Grant number 21267). The authors declare that there is no conflict of interests.

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