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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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 larval 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).

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
hibition of the enzyme activities will prevent para-
site survival. Parasite components such as en-
zymes have specific biological functions, which
are necessary for parasite survival and are sup-
pposed to have an important role in host-parasite
interactions and disease progress (3). Parasite’s
enzymes are attractive purposes that are be ex-
plored 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). Gluta-
thione-S-Transferase (GST) is an enzyme, which
has a significant role in the detoxification of para-
site metabolites (Endogetic), host metabolites
(Xenobiotics) and drugs through their conjugation
to glutathione (5). GST activity in E. granulosus
has been described in the cytosolic portion of proto-
scolecins obtained from sheep cysts and activated
by pre-treatment of protoscoleces with GST in-
ducers (6).

Alkaline phosphates (ALP) is an enzyme that
plays an important role in dampening host im-
mune 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 para-
sites 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 prote-
ase described in E. granulosus (9), has been detect-
ed in hydatid cyst fluid, cyst wall and in protosco-
lecins. 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 pro-
cessing 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 poten-
tial 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 col-
lected 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 Rouz-
darou Pharmaceutical Company (Iran) and ABZ
was purchased from Tolide Daruáhi 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] con-
taining 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 con-
taining 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 protoscoleces 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 µl of buffer reagent (R1) and 200 µl of substrate reagent (R2) were poured into cuvette and mixed. Then 20 µl 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× 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 µl Folin & ciocalteus phenol reagent were added and incubated for 30 min at 37 °C. Finally, tubes were centrifuged for 5 min at 14000× 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/). 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 protoscoleces exposed to ABZ and MB were measured 3.55 and 4.58 µg/ml, respectively. The mean protein concentration in the control group was 6.06 µg/ml.
Table 1: Protein concentration of E/S products of protoscoleces treated and control groups

| E/S product samples | Protein Concentrations(µg/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, repectively. 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/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 activity = \( \frac{\Delta \text{OD}_{0.0096} \times \text{Total volume of sample} \times \text{dilution factor}}{\text{Volume of enzyme} \times \text{Time of assay} \times \text{Volume of filtrate}} \)

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

***Protease activity = \( \frac{\text{umole tyrosine equivalents released}}{\text{Volume of enzyme} \times \text{Time of assay} \times \text{Volume of filtrate}} \)

Specific activity = \( \frac{\text{Units/mg enzyme}}{\text{mg solid}} \)

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 significant 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

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is no significant difference between protein concentration in E/S products of protoscoleces 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 protoscoleces 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 antihydatid 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 antihydatid mechanism of these benzimidazole derivation.

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