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Original Article

Triclabendazole Effect on Protease Enzyme Activity in the Excretory- Secretory Products of *Fasciola hepatica* in Vitro

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Abstract

Background: *Fasciola hepatica* is one of the most important helminthes parasites and triclabendazole (TCBZ) is routinely used for treatment of infected people and animals. Secreted protease enzymes by the *F. hepatica* plays a critical role in the invasion, migration, nutrition and the survival of parasite and are key targets for novel drugs and vaccines. The aim of study was to determine the protease activity of excretory- secretory products (ESP) of *F. hepatica* in the presence of TCBZ anthelmintic.

Methods: *F. hepatica* helminthes were collected and cultured within RPMI 1640 [TCBZ treated (test) and untreated (control)] for 6 h at 37 °C. ESP of treated and control were collected, centrifuged and supernatants were stored at -20°C. Protein concentrations were measured according to Bradford method. Protease enzymes activities of ESP samples were estimated by using sigma's non-specific protease activity assay. ESP protein bands were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results: Mean protein concentrations in control and treated of ESP samples were determined 196.1 ± 14.52 and 376.4 ± 28.20 $\mu\text{g/ml}$, respectively. Mean protease enzymes activities in control and treated were 0.37 ± 0.1 and 0.089 ± 0.03 U/ml, respectively. Significant difference between proteins concentrations and protease enzymes activities of two groups was observed ($P < 0.05$). SDS-PAGE showed different patterns of protein bands between treated and control samples.

Conclusion: The TCBZ reduced secreted protease enzymes activities and possibly effects on invasion, migration, nutrition and particularly survival of the parasite in the host tissues.

Introduction

Fasciola hepatica is the causative agent of liver fluke disease in sheep and cattle and has worldwide distribution especially in the temperate areas (1-4). Furthermore, fasciolosis is a foodborne zoonotic disease in rural areas of developing countries such as Bolivia, Peru, Equador, Egypt and Iran (5). *F. hepatica* larvae penetrate and traverse the intestinal wall, enter the liver capsule, migrate through the parenchyma and cause extensive tissue damage. Finally, parasites complete their growth and maturation in bile ducts (6).

Protease enzymes have an important role in the infectivity and adaptation of *Fasciola* in a wide variety of their hosts (7, 8). These enzymes are secreted by the gastodermis of juvenile and adult flukes. These enzymes are stored in secretory vesicles of the gastodermal epithelial cells as inactive proenzyme. Activation of the proenzyme takes place following secretion into the acidic environment of parasite gut lumen where are needed for food digestion (9). Protease enzymes are secreted by all stages of the developing parasites and are essentially required for various mechanisms necessary for parasitism (10). *Fasciola* spp. release a number of cysteine proteases during their life cycle, including cathepsin L and B proteases (11). Cathepsin protease secreted by the parasite plays a critical role in invasion, migration and survival by cleaving interstitial matrix proteins such as fibronectin, laminin and native collagen, catabolism of host proteins to absorbable peptides and amino acids, and modulation of the host immune response by cleaving immunoglobulin or by altering the activity of immune effector cells. Liver fluke cathepsin L cytosine proteinases are reported as sensitive and specific markers for the diagnosis of human and animal fasciolosis. Cathepsin proteases are also candidate for antiparasitic drugs and vaccines development (12). A number of fasciolicides including

closantel, brotianid, oxyclozanide, clorsulon, rafoxanide, nitroxylnil, diamphenethide, mebendazole, albendazole and triclabendazole have been available for the treatment of animal fasciolosis. TCBZ is a benzimidazole derivative, which have high efficacy against both mature and juvenile stages of *Fasciola* spp., so it is considered as a drug of choice for treatment of liver fluke infections and human fascioliasis. Resistance to TCBZ could severely compromise its future use (13, 14).

In the present study, we have examined the effect of TCBZ on protease enzymes activities of ESP of *F. hepatica* in vitro.

Material and Methods

F. hepatica ESP sample preparation

F. hepatica parasites were obtained from liver of cattle at a local abattoir (Ehsane-Rey, Tehran, Iran). For removing host materials and emptying of the parasite gut, flukes were washed 6 times (1 h) with washing buffer [phosphate buffer saline (PBS): 0.15 M, pH 7.2]. Then, parasites were cultivated for 6 hours in fresh RPMI 1640 (Gibco 51800-019) with additional 10 µm penestrep. TCBZ (Egaten 250mg tablet; Novartis; Switzerland) was initially prepared as a stock solution in dimethyl sulphoxide (DMSO) and 0.6 µl added to the treated culture medium (containing 15 µg/ml concentration TCBZ). In addition, control flukes culture medium was treated with 0.6 µl DMSO. Following incubation, flukes were removed and ESP was collected. So, suspensions were centrifuged at 4 °C, 13000×g for 10 min and were stored at -20°C until required (15-18).

Measurement of ESP samples protein concentrations

The concentration of total proteins of ESP samples were measured according to Bradford assay method which involves reacting the ESP sample with a dye that binds proteins. To

measure of protein concentration, standard solutions (Bovine Serum Albumin, Merk Germany) and ESP samples were prepared and Bradford reagent was added. The absorbance of ESP samples and standard solutions were measured at 595 nm after 10 min incubation at room temperature. A standard curve was prepared using the standard solutions absorbance and the protein concentrations of samples were estimated (18).

Protease Enzymes activities assay of ESP samples

Proteases activity of ESP samples was determined by using sigma's non-specific protease activity assay method. When the protease digests casein as substrate, the amino acid tyrosine is released along with other amino acids. Folin and ciocalteus phenol primarily react with free tyrosine and produce a blue color. Briefly, casein solution prepared and incubated in 37 °C for 5 min, then ESP sample was added to treated tube and incubated for 10 min. Trichloroacetic acid (TCA) added to the treated and control tubes and the reaction was stopped. ESP sample were added to control tubes and incubated for 30min, and then centrifuged for 5 min at 13000×g at 25 °C. Finally, the supernatant was poured into the tube and added sodium carbonate solution and ciocalteus phenol, and was incubated for 30 min at 37 °C. Then, tubes were centrifuged (as above) and tyrosine residues were measured spectrophotometer at 660 nm. The protease activities were compared to a standard curve and reported as Units /ml (19).

SDS-PAGE analysis of ESP samples

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie blue staining were used to separate and stain the components of ESP sample proteins. ESP Samples (30 µg/ml protein concentrations) were mixed with sample buffer and were run on 10% acrylamide gels. Finally, the gel was stained with coomassie blue R-250. Molecular weight of sample proteins in treated samples

and controls were compared with respect to the marker (18).

Statistical analysis

Statistical comparisons were carried out using an online site statistics (<http://www.evansres.com/ttest.html>). Independent *t*-test was performed to compare mean protein concentration and protease enzyme activity between test and control groups (with the confidence interval of 95 %). Results are presented as mean ± SEM.

Results

Protein concentration in control and treated ESP samples

The concentrations of protein in the ESP samples are presented in Table 1 and 2. Mean protein concentrations in control and treated group of *F. hepatica* ESP samples were 196.1 (SE = 14.52) and 376.4 (SE = 28.20) µg/ml respectively.

Proteases enzyme activity in control and treated ESP samples

Protease activity in the *F. hepatica* ESP samples are presented in Table 1 and 2. Mean proteases enzyme activities level in control and treated group were 0.370 (SE = 0.1) and 0.089 (SE = 0.03) U/ml, respectively.

Statistical analysis results of control and treated ESP samples

According to obtained results, significant difference between control group in comparison to the treated ESP samples in protein concentrations [*T* (18) = 5.84, *t*-value = 2.1] and proteases activities [*T* (18) = 2.52, *t*-value = 2.1] was observed (*P*<0.05).

SDS-PAGE results of treated and control ESP samples

Protein of ESP samples were analyzed by SDS-PAGE electrophoresis and are shown in Fig. 1. There are qualitatively differences in

protein bands between SDS-PAGE results of treated and control ESP samples. Protein

bands 146, 96 and 77 were observed in treated samples but are not seen in control samples.

Table 1: Protein concentrations and proteases enzyme activities in control ESP samples of *F. hepatica*

ESP Samples	Protein Concentrations (µg/ml)	Proteases enzyme activities (U/ml)
S1	196	0.478
S2	189	0.799
S3	130	0.534
S4	181	1.073
S5	181	0.277
S6	276	0.148
S7	140	0.148
S8	185	0.166
S9	229	0.088
S10	254	0.047
Mean	196.1	0.370

Table 2: Protein concentrations and proteases enzyme activities in treated ESP samples of *F. hepatica*

ESP Samples	Protein Concentrations (µg/ml)	Proteases enzyme activities (U/ml)
S1	358	0.036
S2	351	0.028
S3	449	0.100
S4	400	0.004
S5	282	0.004
S6	367	0.325
S7	392	0.084
S8	560	0.237
S9	229	0.072
S10	376	0.006
Mean	376.4	0.089

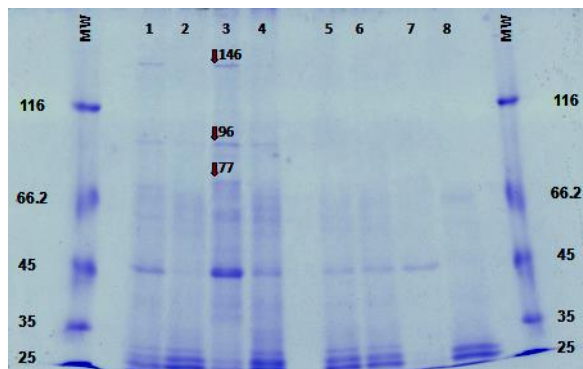


Fig. 1: SDS- PAGE analysis of proteins from the ESP samples *F. hepatica*. The treated samples (containing TCBZ) were presented in lane 1-4 and the

control samples in lane 5-8. Protein bands 146, 96 and 77 were observed in treated samples

Discussion

In the present study, the effect of TCBZ on ESP protein of *F. hepatica* was examined. Protein concentrations in treated ESP samples were more than control ESP samples and show the tegument and viteline cells were probably disrupted by TCBZ (20-22).

Proteases enzyme activity in treated ESP samples was less than control samples. Since protease enzymes are proteins, reduction pro-

tease enzymes activity could be due to interference of TCBZ with protein synthesis (23). In S3, S6 and S8 treated samples, enzyme activity reduction were less than other samples because primary enzyme level of individually parasite was probably higher than others. Bennett and Kohler showed that the TCBZ reduces the secretory protease enzyme and stated that likely TCBZ's obvious ability to strongly bind to various kinds of proteins including microtubules and leads to decrease enzyme activity (24).

Proteases play an important role in the evasion from host immune system. On the one hand, suppresses the Th1 lymphocytes and propels the immune system toward Th2 cause to prevent the acute phase of illness. On the other hand, cleaving immunoglobulin (25, 26), prevents antibody-mediated eosinophil attachment to newly hatched juveniles (24) and eosinophil apoptosis (27). Due to the reduction of protease enzymes by TCBZ and with attention to roles of proteases on the immune system can be concluded that one of the effects of TCBZ is probably restoring the immune system. Hence, proteases can inhibit the immune system and controversially the TCBZ reduces the activity of protease enzymes, so resulting reduction in immunosuppression and staying active that can be an important step in eliminating the parasite. Then, TCBZ treatment has direct effect on the *F. hepatica*, by destruction of parasite tegument, also reduce of proteases activity and recover immune system indirectly.

The results of SDS-PAGE gel of *F. hepatica* ESP show protein bands with different molecular weights in the control and treated samples. In the treated samples protein bands with different molecular weights (146, 96 and 77 kDa) can be seen without existing in control group. The mentioned protein bands were probably released from parasites due to destruction of tegument, but need to verify by qualitative methods in more researches.

Conclusion

TCBZ increases protein concentrations, protein bands and reduces the proteases enzyme activities in ESP of *F. hepatica*. The TCBZ reduced secretory protease enzymes activities and possibly effects on invasion, migration, nutrition and particularly survival of the parasite in the host tissues.

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