Activity Assay of Glutathione S-Transferase (GSTs) Enzyme as a Diagnostic Biomarker for Liver Hydatid Cyst in Vitro

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Abstract
Background: The aim of this study was to detect the Glutathione S-Transferase (GST) enzyme activity of healthy / cystic liver as a diagnostic biomarker for hydatidosis. In order to compare with liver tissue, the level of the GSTs enzyme activity of parasite was also determined.
Methods: Parasites were collected from sheep liver tissue with hydatid cysts at a local abattoir and washed with PBS buffer. Collected parasites and liver tissues were sonicated or homogenized respectively. Extract solution samples were centrifuged and stored at -20°C. GST enzyme activities were measured in the extract of parasite and liver tissue samples (healthy and infected livers). Protein amounts and protein bands were detected using Bradford and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods respectively. To determine significant difference between two groups, two-sample t-test was performed.
Results: GST specific activities of healthy / infected livers and parasites were estimated 304, 1297 and 146 U/ml/mg respectively. Significant higher GST specific activities in cystic liver than healthy liver was observed (P<0.05). T-test analysis showed GST activity of parasite was lower than healthy liver tissue. SDS-PAGE showed GST protein bands with 24 kDa in parasite samples and 25 kDa in liver tissues.
Conclusion: GST activity in cystic liver tissue could be concerned as a biomarker for hydatid cyst diagnosis with other hydatid disease parameters.

Keywords: Glutathione S-Transferase, Hydatid cyst, Protoscolices, Liver, Parasite

Introduction

Infection with Echinococcus parasite may be naturally transmitted between humans and animals. Hydatidosis term used to refer to infection with the parasite larva in humans and echinococcosis restricted to infection with the adult stage in carnivorous animals (1). Recently, the genetics, structure and function of the human cytosolic GST enzyme with emphasis on their roles in the cellular metabolism has been defined (2). This enzyme protect cells against toxicants by conjugating the glutathione as substrate to xenobiotics. GST activity was detected in most mammalian tissues, especially in the liver which plays a key role in detoxification. There are different classes of GST isozymes that diverge in their specificity to xenobiotic or endogenous substrates (3). Enzymes are essential for survival, migration and metabolism of parasites. GST enzymes involved in the cellular detoxification of a broad range of chemical substrates (4). Apart from reac-
tion from their endogenous metabolism, GSTs of helminth parasite may protect against exogenous xenobiotics as a result of immune effectors mechanisms from the host (5). Glutathione transferase activity has been determined in cestodes, digenecas and nematodes. Significantly higher activity has been found in intestinal cestodes and digenecas, compared with parasitic nematodes (6). GSTs activity assay has been demonstrated in the cytosol of protoscolices from sheep hydatid cysts (7). The liver is the most common organ involved by hydatidosis (8). Diagnosis of hydatid disease is done by a combination of clinical signs, imaging techniques, cyst fluid examination, serological tests and molecular techniques (9). The prevalence of liver hydatid cyst was reported 4.7% in people of Peru country using recombinant antigen, EpC1 glutathione S-transferase [rEpC1-GST], in western blot technique (10). However, the hydatid cyst diagnosis technique is under developing due to specificity and sensitivity problems. In the present study, GST enzyme activity of hydatid cyst protoscolices (parasite), healthy and cystic liver tissues were compared and mentioned GST enzyme importance for diagnostic biomarker in hydatid cyst disease.

Materials and Methods

Preparation of protoscolices (parasite) extracts solution

Ten samples of parasites were obtained from 10 liver infected with hydatid cysts of sheep slaughtered at a local abattoir (Karaj, Iran). Parasites samples were washed 3 times with PBS buffer, pH 7.2, freeze-thawed 3-6 times in liquid nitrogen and water bath 37°C respectively and sonicated in a 150W ultrasonic disintegrator, 10 sec ON and 5 sec OFF on ice until no intact PSC were visible microscopically (approximately 15 min). Then resulted suspension was centrifuged (10000g for 30 min at 4°C) and supernatant was stored at -20°C (11).

Preparation of Liver extracts solution

Sheep livers (10 health liver samples and 10 infected samples) were obtained at a local abattoir and washed 3 times with PBS buffer pH 7.2. Then they were homogenized with 3 volumes of homogenizing buffer, PBS pH 6.5, in a glass homogenizer, so the suspension were centrifuged (10000g for 30 minat 4°C) and supernatant stored at -20°C (12).

Protein assay in the solutions

The protein concentration in the extract solutions of protoscolices and sheep liver tissues were estimated by the method of Bradford using bovine serum albumin as the standard (12).

GSTs activity assay in the solutions

GSTs activity was assayed spectrophotometrically at 25°C with reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates. This was done by watching an increase in absorbance at 340nm. Protosolices and liver extract samples were removed from -20°C freezer and allowed to thaw on ice. CDNB 100 mM from 4°C and GSH 100 mM from -20°C freezer were removed and allowed to thaw at room temperature, when thawed, incubated at 30°C in water bath. For each assay was prepared one ml of assay cocktail (980μl PBS pH 6.5, 10μl of 100 mM CDNB and 10 μl of 100 mM GSH), then removed 100 μl of cocktail and its remaining placed 900 μl of it into 1.5 ml cuvette. To zero spectrophotometer, was used 1 ml of distilled water and to the blank cuvette added 100μl PBS to 900 μl of cocktail and measured absorbance at 340 nm, every 1 minute, for 3 min. To the test cuvette was added 100 μl of sample to 900 μl cocktail, mixed and measured absorbance at 340 nm as above (13).

SDS-PAGE analysis of samples

SDS-PAGE and coomassie blue staining were used to separate and stain the protein components of samples respectively. Samples were mixed with sample buffer and were run on 10% acrylamide gels. Finally, the gel was stained with coomassie blue R-250. Molecular weights of sample proteins were compared with respect to the protein marker (12). To detect the molecular weight, Rf(Ratio factor) of ladder bands was calculated, standard curve

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was prepared in Excel software and finally proteins MW were determined. The proteins of gels were identified primitively by using protein database (http://web.expasy.org).

**Statistical analysis**

Independent t-test was performed to compare the mean values of protein concentration and enzyme activity between healthy and cystic liver tissues or parasite and liver tissues. Statistical comparisons were carried out using statistical software (14).

**Results**

**Protein concentration, enzyme activities and statistical analysis results**

The mean values of protein concentrations and enzyme activities for parasite, healthy and infected liver samples are presented in Table 1.

### Statistical test

Statistical t-test showed GST enzyme activity of parasite was lower than healthy liver tissues ($P<0.05$).

### SDS-PAGE analysis results

Extract samples of protoscolices, healthy and cystic liver tissues were analyzed by SDS-PAGE electrophoresis and the results are shown in Fig. 1. SDS-PAGE gel shows GST protein bands with 24kDa in parasite and 25kDa in healthy liver samples. Similar cross protein bands is observing in parasite and liver sample but has been not recorded by database. Identified proteins are presented in table 2, 3.

### Table 1: The mean values of protein amounts and GSTs activity for parasite, healthy and cystic liver samples

| Samples          | Protein amounts (mg/ml) | GST Total activity (U/ml)* | GST Specific activity(U/ml/mg/protein) |
|------------------|-------------------------|---------------------------|----------------------------------------|
| Healthy liver    | 5.0±0.7                 | 1522±0.27                 | 304.4±0.09                             |
| Cystic liver     | 2.3±0.5                 | 2984±0.61                 | 1297±0.16                              |
| Parasite extract | 0.1±0.09                | 14.60±0.0                 | 146±0.0                                |

*One unit of GST activity is the amount of enzyme which produces 1 µmol of GS-DNB conjugate/min under the conditions of the assay.

[Fig. 1: Molecular weight of proteins (kDa) from the extracts of parasite and liver tissues in SDS-PAGE. Lanes 1-3 are healthy liver samples. Lanes 4-6 are cystic liver samples. Lanes 7-9 are parasite samples (Protein marker, peqGOLD I, Lot-No. 64072)]
### Table 2: Identified proteins of parasite according to molecular weight by using protein database (http://web.expasy.org)

| MW according to Ratio factor (Rf) | MW according to database | Protein                                                                 |
|----------------------------------|--------------------------|--------------------------------------------------------------------------|
| 80.537                           | 84.086                   | Acetyl choline transferase                                                |
|                                  | 77.460                   | SmD eD                                                                   |
| 75.857                           | 72.271                   | Phosphoenolpyruvate carboxylase                                          |
|                                  | 75.392                   | UDP-N-acetyl-D-galactosamin: N0acetyl Galactosaminyltransferase           |
| 65.012                           | 65.213                   | AntigenII/3                                                              |
|                                  | 65.261                   | CYtoskeleton/extrinsic tonembrane (eq10)                                  |
|                                  | 65.465                   | TGR Thioridoxinreductaseserin/Threonine-protein Phosphatase              |
|                                  | 66.310                   |                                                                          |
| 59.429                           | 59.187                   | NADHdehydrogenase subunit5                                              |
|                                  | 59.222                   | ALP                                                                      |
|                                  | 60.100,60.200,60.242,60.300 | Glutamyl – transferase                                                   |
|                                  | 60.301,60.304,60.318     |                                                                          |
|                                  | 61.140                   |                                                                          |
| 52.751                           | 51.389                   | RNA poly II (rpb2)                                                       |
|                                  | 54.705                   | AMP activated protein kinase - 4                                        |
|                                  | 54.714                   | PhosphoenolpyruvateCarboxylase (pepk)                                   |
|                                  | 54.849                   | AMP activated protein kinase - 4                                        |
|                                  | 54.875                   | Ag5 (Serin-type endopeptidase activity)                                  |
| 47.863                           | 45.978                   | NASH-ubiquinoneroxidoreductase chain 4 (ND4)                             |
|                                  | 46.561                   | Enolase                                                                  |
|                                  | 46.689                   | Antigen EG13                                                             |
|                                  | 47.782                   | NADH-ubiquinone                                                          |
|                                  | 48.857                   | Isodredutaset chain 4                                                    |
|                                  | 48.857                   | Elnogation factor 1 (rfla)                                              |
| 23.067                           | 22.571                   | NADH-ubiquinone                                                          |
|                                  | 22.573                   | Oxidoreductase chain 1 (ND1)                                             |
|                                  | 23.050                   | NADH-ubiquinone                                                          |
|                                  | 23.881                   | Oxidoreductase chain 1 (ND1)                                             |
|                                  | 24.147                   | Ra I-like protein                                                        |
|                                  | 24.226                   | COX3                                                                     |
|                                  | 24.301                   | Potative cysteine peptidase                                              |
|                                  | 24.301                   | GST2                                                                    |
| 19.230                           | 18.382                   | Arginine N-methyl                                                       |
|                                  | 18.502                   | Transtensel                                                             |
|                                  | 18.991                   | Actin-1 (ACT1)                                                           |
|                                  | 19.046                   | Calcinurine B (calB)                                                     |
|                                  | 19.356                   | Calcium B-like protein                                                   |
|                                  | 20.046                   | 22KDa antigen 5                                                          |
|                                  | 20.705                   | ATPase subunit6                                                         |
|                                  | 19.733                   | ATP synthase F subunit6                                                  |
|                                  | 19.747                   | NDI (ap6)                                                                |
|                                  | 19.881                   | CO1                                                                      |

### Table 3: Identified proteins of sheep liver tissue according to molecular weight by using protein database (http://web.expasy.org)

| MW according to Ratio factor (Rf) | MW according to database | Protein                                                                 |
|----------------------------------|--------------------------|--------------------------------------------------------------------------|
| 65.012                           | 65.675                   | Betaacronen oxygenase2                                                   |
|                                  | 65.235                   | Polactin receptor (PRL-R)                                                |
| 59.429                           | 59.230                   | Coatomer Protein Complex4                                                 |
|                                  | 59.230                   | Cytochrom P4501A1(CYP1A1)                                                |
| 52.751                           | 52.026                   | NADH dehydrogenasesubunitne4                                             |
|                                  | 52.970                   | 6phosphogluconate dehydrogenase                                         |
|                                  | 53.025                   | Serine hydroxyl methyl transferase                                       |
| 47.863                           | 45.983                   | alpha-1 anti proteinase, alpha-1 anti trypine                            |
|                                  | 46.716                   | Thyroid hormone receptor beta                                            |
|                                  | 48.056                   | Corticosteroid-binding globuline                                         |
|                                  | 48.056                   | Serpin A6, Transcottin                                                   |
| 23.067                           | 23.127                   | Secreted phosphoprotein24                                                |
|                                  | 23.601                   | Copperphaperone of SODI                                                  |
|                                  | 23.608                   | Catheitin L1 (CTSL)                                                      |
|                                  | 25.244                   | GST                                                                      |
| 19.230                           | 19.121                   | NADH – ubiquinone oxidoreductase chain 6                                 |
|                                  | 19.842                   | Cathelicidins2, (Bactenecine-5)                                          |
|                                  |                         | (Bac5),(CaTHL2)                                                          |
Discussion

Liver tissue is the most important source for protein synthesis and detoxification. Two major types of liver cells are hepatocytes and sinusoid cells. Function of hepatocytes may be disturbed in the presence of infections (15). In our study the protein concentration of infected tissue was reduced. The reduction of protein synthesis as a result of hydatid cyst causes to decrease protein concentration. GSTs can make up to 10% of cytosolic protein in some mammalian organs (16). Hepatic cells contain high levels of GST enzyme which has been found to be an indicator of hepatocyte injury in transplantation, toxicity and infections (17). The hydatid cyst infection stimulates oxidative stress and toxin production in hepatic cells. From the point of biochemical defense view GST enzyme be able to neutralization of these toxins, therefore we expect to increase activity level of this enzyme. Increase of liver protein and GST in infected mice indicates the occurrence of oxidative stress in hepatocytes due to infection (18). α-glutathione S-transferase (GSTA) is distributed homogeneously in the liver tissue. Serum GSTA is a more sensitive marker than transaminases (Alanine aminotransferase, Asparatate aminotransferase and Alkaline transferase) for monitoring and as an early analyst of hepatic damage (19). Therefore GST activity difference between healthy and infected host liver tissue could be concerned for hydatid cyst diagnosis. However, other infections cause to hepatocyte damage, thus GST elevation must be evaluated with other clinical and paraclinical parameters of hydatid cyst disease.

Mammalian cytosolic GSTs are dimeric, with both subunits being from the same class of GSTs, although not necessarily identical. The monomers are approximately 25 kDa in size (20). GST enzyme molecular weight of parasite is reported 24-27 kDa (13). In this research protein bands with 24kDa and 25kDa were found in parasite and liver tissues respectively. This protein is very important from parasite survival point of view.

Conclusion

GST activity in cystic liver tissue could be concerned as a biomarker for hydatid cyst diagnosis with other parameters of hydatidosis.

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